Improvement of Functional Properties of Soy Protein

By

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Abstract

The objective of this thesis was to study the effect of heat treatment and glycation on five industrially important functional properties of soy protein, namely solubility, emulsifying ability, water holding ability, acid gelation ability in soy yogurts and the heat stability of soy protein emulsions.

These objectives were achieved completing three tasks:

1) The physicochemical properties were studied of Soycomil K, a commercially available, insoluble soy protein concentrate. Differential scanning calorimetry (DSC) analysis showed that it was 28% denatured compared to native, laboratory SPC. Further analysis showed that the aggregated structure is more hydrophobic and there are more disulfide bonds than found in commercially available soy protein isolates (SPI). Heat treatment at alkaline pH and low protein concentration increased its solubility. Heat treatment at 100 °C increased SoyComil's solubility significantly compared to heat treatment at 70 °C. Glycation of SoyComil K with glucose at 70 °C increased solubility compared to the control, whereas glycation at 100 °C decreased solubility. Heat treatment of Soycomil increased its solubility more than glycation.

2) A soy yogurt with smooth texture and high water holding capacity (WHC) was developed with SPI heated in the presence of pectin and glucose, followed by homogenization with the oil ingredient of the yogurt recipe. The texture matched that of commercially available yoghurt manufactured with soymilk. A yogurt made with SPI only showed low WHC. These results provide evidence that combined heat treatment and glycation of SPI improved its functional properties. A study of the structure of the yogurt showed that the majority of bonds were hydrophobic bonds, whilst electrostatic and disulfide bonds played a small part in maintaining the yogurt structure.

3) A study of the rate of aggregation of SPI stabilized emulsions at pH4.5 showed that the heat stability of SPI emulsions was strongly dependent on protein concentration and temperature. A second study showed that the presence of polysaccharides either improved or had little effect on heat stability depending on the concentration of polysaccharide added. This was attributed to interactions between soy proteins and specific polysaccharides. A study of the heat stability of mixed whey protein concentrate (WPC) and SPI emulsions showed that WPC dominated the oil droplet destabilization behaviour, and that low proportions of WPC were able to slow down the heat-induced breakdown of SPI/WPC-emulsions containing a high proportion of SPI.

Dedication

To my dear late father, Ali, who was like a burning candle giving me light. He taught me that a person who has done good science and honourable work in his life will be immortal. I dedicate my whole work to his gracious soul.

ACKNOWLEDGEMENTS

It is with great pleasure that I write this section of my thesis. In my heart and mind, it is the most important part of this thesis. I get to say "thank you" to all the wonderful people who have made it possible for me to go through this program. My deepest gratitude is to Allah my maker. I am extremely thankful to Allah for blessing me with life, a good health, the intellect and resources to be able complete this program. In my most difficult moments, the word of Allah always served to up life my sprit, and give me a sense of purpose.

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DECLARATION

I, Al-Amari Ali Al-Bakkush, hereby declare that I am the author of this thesis. All the work described in this thesis is my own except where stated in the text. The work presented here has not ben accepted in any previous application for a higher degree. All the source of information have been consulted by myself and are acknowledged by means of refrence.

(Al-amari Al-Bakkush)

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Abbreviations:

ANS: 1-anilino-8-naphtalene sulfonate

AOAC: The Association of Official Analytical Chemists

Bis-Tris: Bis(2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl

BSA: Bovine Serum Albumin

CSLM: Confocal scanning laser microscopy

Cys: Cysteine

dH_2O: Distilled H_2O

DSC: Differential scanning calorimetry

DTNB - 5,5′-dithio-bis(2-nitrobenzoic acid)

EDTA: Ethylenediaminetetraacetic acid

GDL: Glucono-δ-lactone

M: Molarity

MW: Molecular Weight

OPA: *O*-phthaldiadehyde

O/W: Oil in Water emulsion

PAGE: Polyacrylamide Gel Electrophoresis

pH: Power of Hydrogen

pI: Isoelectric point

α-lac: α-Lactoalbumin

β-lac: β-Lactoglobulin

RI: Refractive Index

RT: Room temperature (22 ± 3°C)

Rpm: Rotations per Minute

SDS: Sodium Dodecyl Sulfate

SH: Sulfhydryl group

SPC: Soy protein concentrates

SPI: Soy protein isolates

TCA: Trichloroacetic Acid

TPA: Texture profile analysis

WHC: Water-holding capacity

WPC – Whey Protein concentrate

Chapter One

General introduction

General Introduction

1.1 Introduction

The soybeans family Leguminosae is a native crop of eastern Asia. Domestication of the soybean is believed to have originated in the northern and central regions of China as long as 5000 years ago, with the first documented use of the plant by a Chinese emperor. Soybean cultivation spread throughout Japan, Korea, and Southeast Asia, although the USA and Brazil account today for most of the soybean production of the world. Soybeans are, primarily, an industrial crop, cultivated for oil and protein (Berk, 1992). As the world population expands, there will be a greater pressure for the consumption of plant products (Kinsella, 1979). Today soybeans are one of the most economical and valuable agricultural commodities because of its unique chemical composition and multiple uses as food, feed and industrial materials. Soybeans have the highest protein content among cereal and other legume species, and the second highest oil content among all food legumes. Soy protein contains most the essential amino acids, most of which are present in amounts that closely match those required for humans or animals. Furthermore, soybeans also contain many biological active components, including isoflavones, lecithin, saponins, oligosaccharides, and phytosterols. Many of these components act as anti-cancer agents and antioxidants (Liu, 2004). Currently there is considerable and increasing interest in the health benefits of soy-containing foods, in particular in the role of soy protein in lowering the incidence of certain cancers. It has been suggested that the high intakes of soy may explain, in part, the lower incidence of certain cancers in Asian countries, where soy consumption is high, when compared to Europe or America (Davies *et al*., 1998). Due to its nutritional value and low cost, currently, soy protein is the largest commercially available vegetable protein in the world, and it is an important alternative to existing animal derived proteins. Soy proteins are also of particular interest because they impart high functionality in food systems, being used to obtain better quality products. Because of these advantages (economic, nutritive, dietetics, etc.) it is important to develop new soy protein foods or a range of new food formulations with new textures (Molina *et al*., 2002).

1.2 Soybean structure and composition.

Soybeans vary from almost spherical to elongated and flat. The colour of the seed may be yellow, green, and brown or black, comprising of about 8% hull (seed coat), 90% cotyledon, and 2% hypocotyl (Berk, 1992).

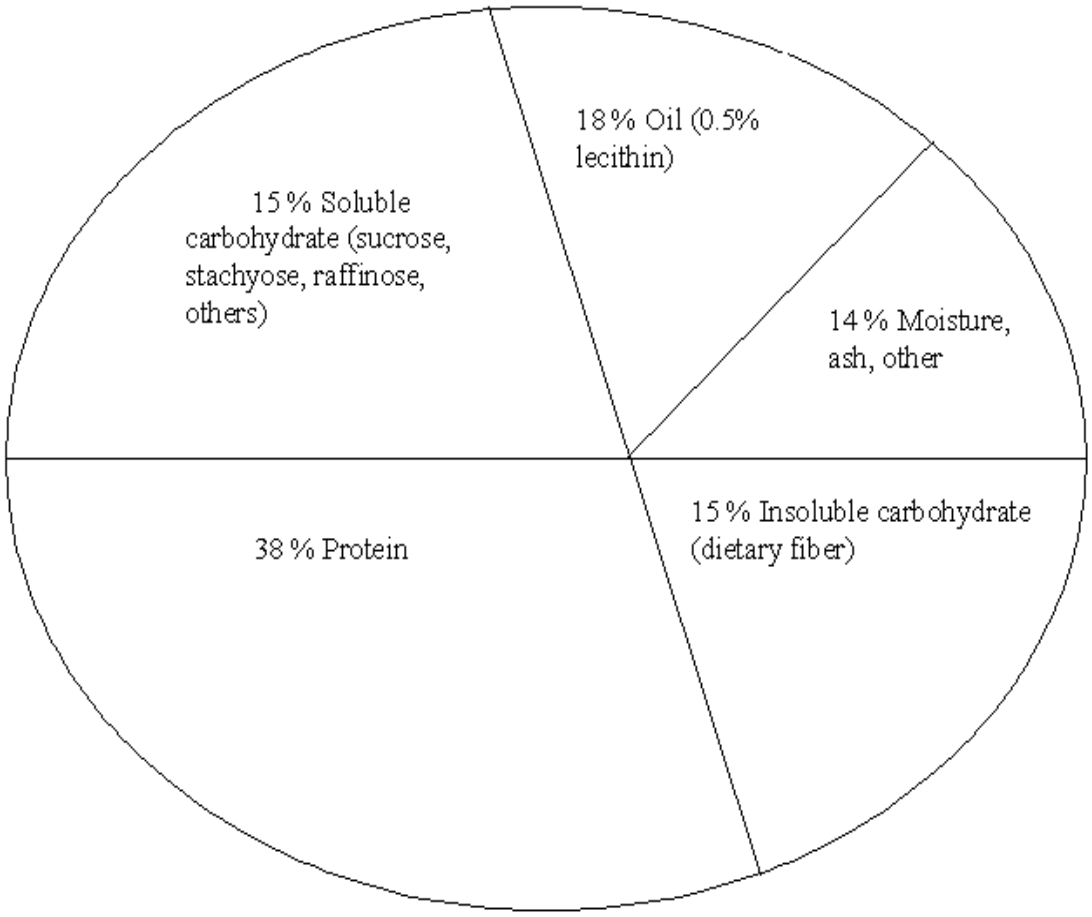


Figure 1.1. Chemical composition of soybean (Kuen , 2004)

1.3 Essential amino acid composition of soybean

The structural units of proteins are amino acids, which are necessary for growing and maintenance of human body. Soy protein contains all the essential amino acids except methionine and tryptophan (Russel, 2004). Soy proteins are high in lysine and thus are useful supplements for cereals, which tend to be low in this amino acid. On the other hand, methionine is the first limiting amino acid in soy proteins and this limitation must be

considered when the proteins are added for nutritional purpose rather than simply for functionality (Wolf, 1970 and Morita *et al* 1997).

1.4 Components of soy protein

Soy protein contain a broad range of components that is classified in terms of Svedburg sedimentation units, S. The smaller the Svedburg number, the smaller the protein molecular weight (Kuen, 2004). Soy protein may be grouped into four categories as follows:

1.4.1 2S fraction (whey protein)

This fraction has been reported to contain 15-22% of soy protein with molecular weight ranging from 8 to 21.5 KDa. It consists of number of enzymes, which are trypsin inhibitors and cytochromes (Wolf, 1970; Tay *et al*, 2005). The 2S fraction is a highly symmetrical protein composed of a number of tight rings. These rings are held together by 7 disulfide bonds (Kuen, 2004). The 2S fraction exhibits good foaming capacity and water holding ability (Tay *et al.*, 2006).

1.4.2 7S fraction (β-conglycinin)

β-Conglycinin (7S) is one of the dominant storage proteins of soybean seeds, constituting about 30% of total soy protein. It is a trimeric protein with molecular weight of 180 – 210 KDa (Maruyama *et al*., 2002; Wolf, 1970). The 7S fraction contains approximately 85.06-90.17% protein, 1.38-1.96% fat, 4.8-5.91% ash, 1.82-8.81% carbohydrates and traces of fiber (Khatib *et al*, 2002). The protein is composed of three subunits, α (~67 KDa), α' (~71 KDa), and β (~ 50KDa). Several combinations of these subunits (α' β2, α β2, α α'β, α 2α', α3 and β) are known to exist and provide heterogeneity (Maruyama *et al*, 2002; Rickert *et al*., 2004). The α and α' subunits are composed of extension regions and core regions and have acidic properties, whereas the ß subunit consists of only the core region (Maruyama *et al*, 2002; Rickert *et al*., 2004). The thermal solubilities of individual subunits are different indicating that that they exhibit different physicochemical functions (Maruyama *et al,* 1999). B-conglycinin has many properties, one of it is its ability to form disulfide – linked polymers, which contribute to insolubility of soy protein. These polymers cause turbidity and increased viscosity of soy protein

dispersions. Depolymerization can be achieved by adding of mercaptoethanol. A second property is sensitivity to ionic environment; it undergoes association- dissociation reactions with change in ionic strength.

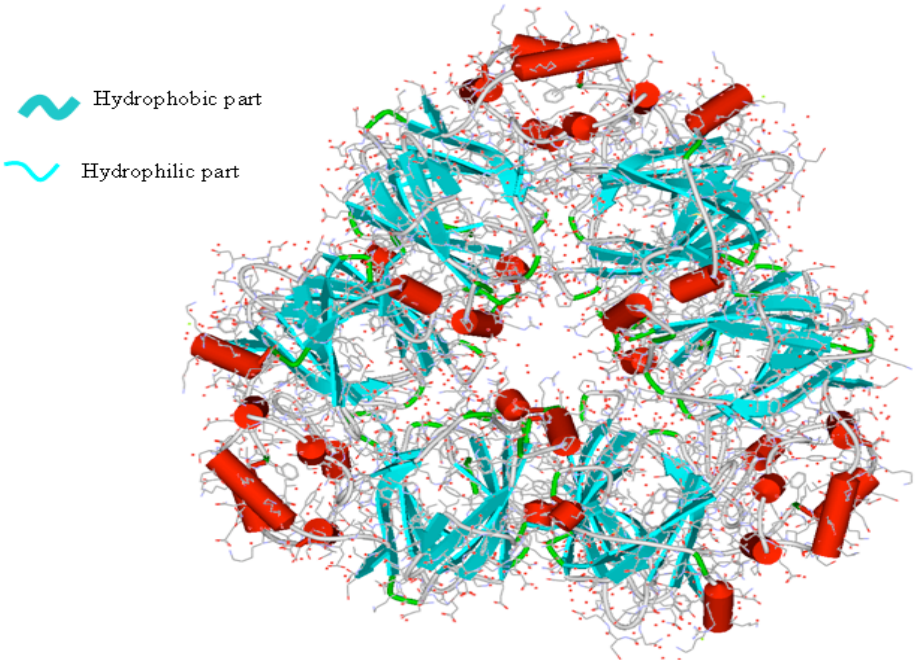
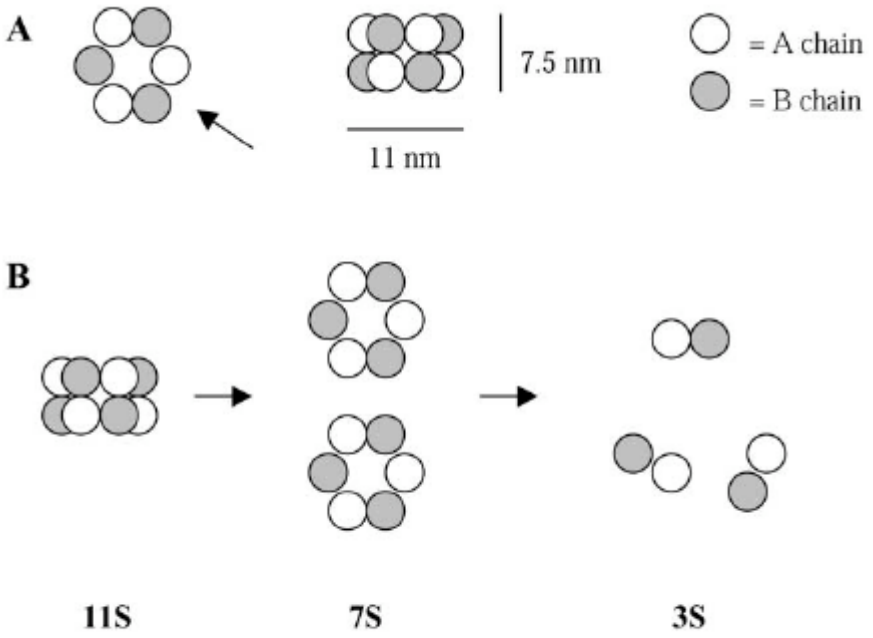


Figure 1.2. The ribbon diagram of native soy β-conglycinin (Barać *et al.*, 2004)

1.4.3 11S fraction (glycinin)

Glycinin is one of the major constituents of soy protein representing ~ 35% of total protein (Utsumi *et al.*, 1987; Lakemond *et al*., 2000b and Tay *et al*., 2005). Native glycinin is a heterogeneous oligomeric protein, the molecular mass of which varies between 340-375 kDa. It is made up of six subunits, each consisting of a basic polypeptide (B-polypeptide, MW 38 KDa) and acidic poly peptide (A- polypeptide, MW 20KDa), which are connected by a single disulfide (SS) bond forming AB subunits (Utsumi *et al*.,1987; Renkema *et al*.,2000; Petruccelli and Añón,, 1994 and Petruccelli and Añón, 1996). The AB subunits associate into two hexagonal rings forming a hollow cylinder of 11 x 11 x 7.5 nm (Figure 1.3) (Martin *et al.*, 2002). At ambient temperature and at pH 7.6, glycinin forms hexameric complexes, whereas at pH 3.8 glycinin is mainly present as trimeric complexes (Lakemond *et al*., 2000a). Glycinin contains extensive disulfide bonds, which contributes to

its insolubility. It undergoes association- dissociation reactions, with changes with ionic strength. The quaternary structure of the 11S molecule is disrupted by high and low pH, by high concentrations of urea, detergents, mercaptoethanol-urea mixtures and by temperatures above 80°C (Wolf, 1970). The onset denaturation of glycinin is about 80°C at neutral pH (Renkema and Van Vliet, 2002). Thermal aggregation of glycinin at 80°C is mainly due to aggregation of the basic subunits after they have been thermally dissociated from the oligomeric structure. This aggregation is prevented in the presence of the other soy protein fractions. This is because of certain interactions between conglycinin and the basic subunits of glycinin, which may lead to formation of soluble complexes (Damodaran and Kinsella, 1982).



(a) structure of (soy) glycinin; (b) dissociation of glycinin.

Figure 1. 3. Schematic drawing of the structure of glycinin (Martin et al., 2002)

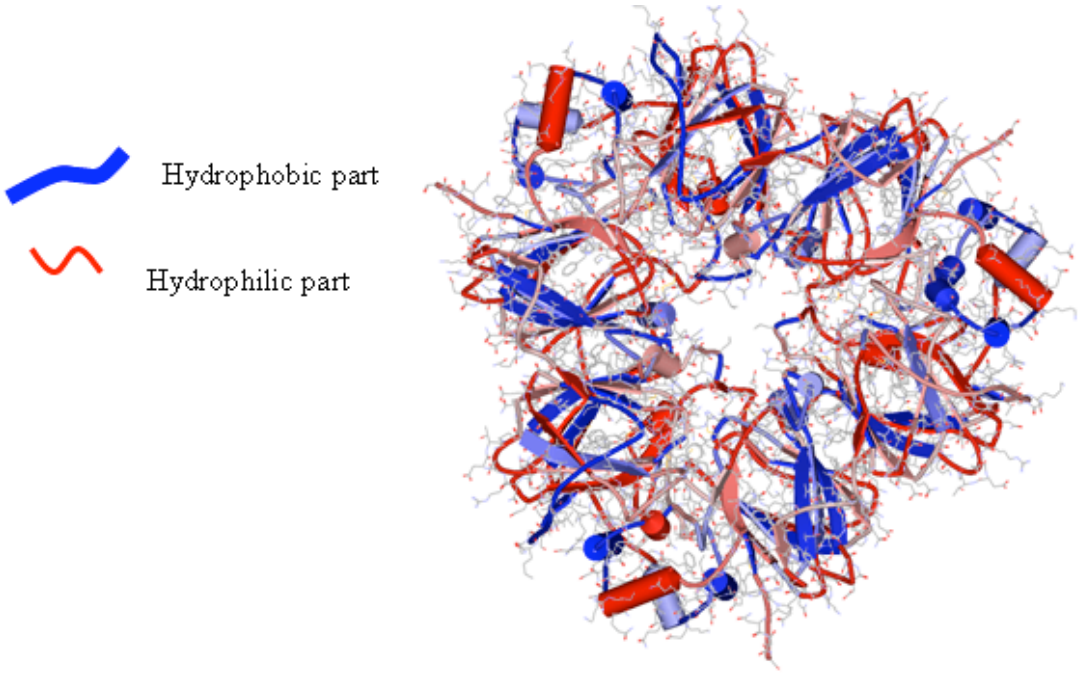


Figure 1.4. The ribbon diagram of native soy glycinin (Adachi , 2003)

1.4.4 15S fraction

The 15 S is a minor component of soy protein (11%), with molecular weight of 506- 600 kDa. 15S either exists as a native protein in the seed or is an artefact formed during isolation of the proteins (Wolf and Nelsen, 1996; Kuen, 2004). Conversion of 11S to 15S fraction occurs when 11S preparations are frozen and thawed or precipitated by dialysis against water and freeze-dried (Wolf and Nelsen, 1996).

1.5 Commercial soy products

In industry, three different soy products are used: soy flours, soy concentrates and soy isolates**.**

1.5.1 Soy flours

Soy flours are produced from soybean flakes by grinding and screening after or before extracting the oil, with protein concentration from 40 to 54%. Soy flours vary in fat content and particle size depending on different degrees of heat treatment and are less refined than other soy forms used for human and animal consumption,. It can also be

obtained as lecithinated or re-fatted forms (Wolf, 1970 and Endres, 2001 and Olaoye *et al*., 2006). Soy flours consist approximately of 6-8% moisture, 40-54% protein, 0.5-1% fat, 30-32% carbohydrate and 5.0-6.0% ash (Endres, 2001). Flour carbohydrates consist of a soluble fraction: oligosaccharides (sucrose, stachyose and raffinose), and an insoluble fraction: cellulose, hemicellulose, pectin and trace amount of starch (Wolf, 1970 and Liu, 2004).

1.5.2 Soy protein concentrates

Soy protein concentrate (SPC) contains approximately 5% moisture, > 66% protein, <1% fat, 23% carbohydrate, and 5% ash (Stauffer, 2002). Traditionally SPC is obtained from defatted soy flakes or flour. SPC is obtained by removing soluble sugars and oligosaccharides (short polymers of several monosaccharides joined by glycosidic bonds; Nelson, 2008) and minor constituents from the defatted flakes using aqueous alcohol or a dilute acid solution in the pH range of 4.0-4.8 as shown in Figure 1.5 (Wang *et al*., 2004 and Alibhai *et al*., 2006). The functional properties such as solubility and water binding ability of commercial SPC's vary greatly, and are determined by their individual extraction procedures. In this study, the properties of a particularly insoluble commercially available SPC, Soycomil K, were studied. It is manufactured and sold as animal feed by Archer Daniels Midland Company, Netherlands (ADM). Its manufacturing process is shown in Figure 1.6.

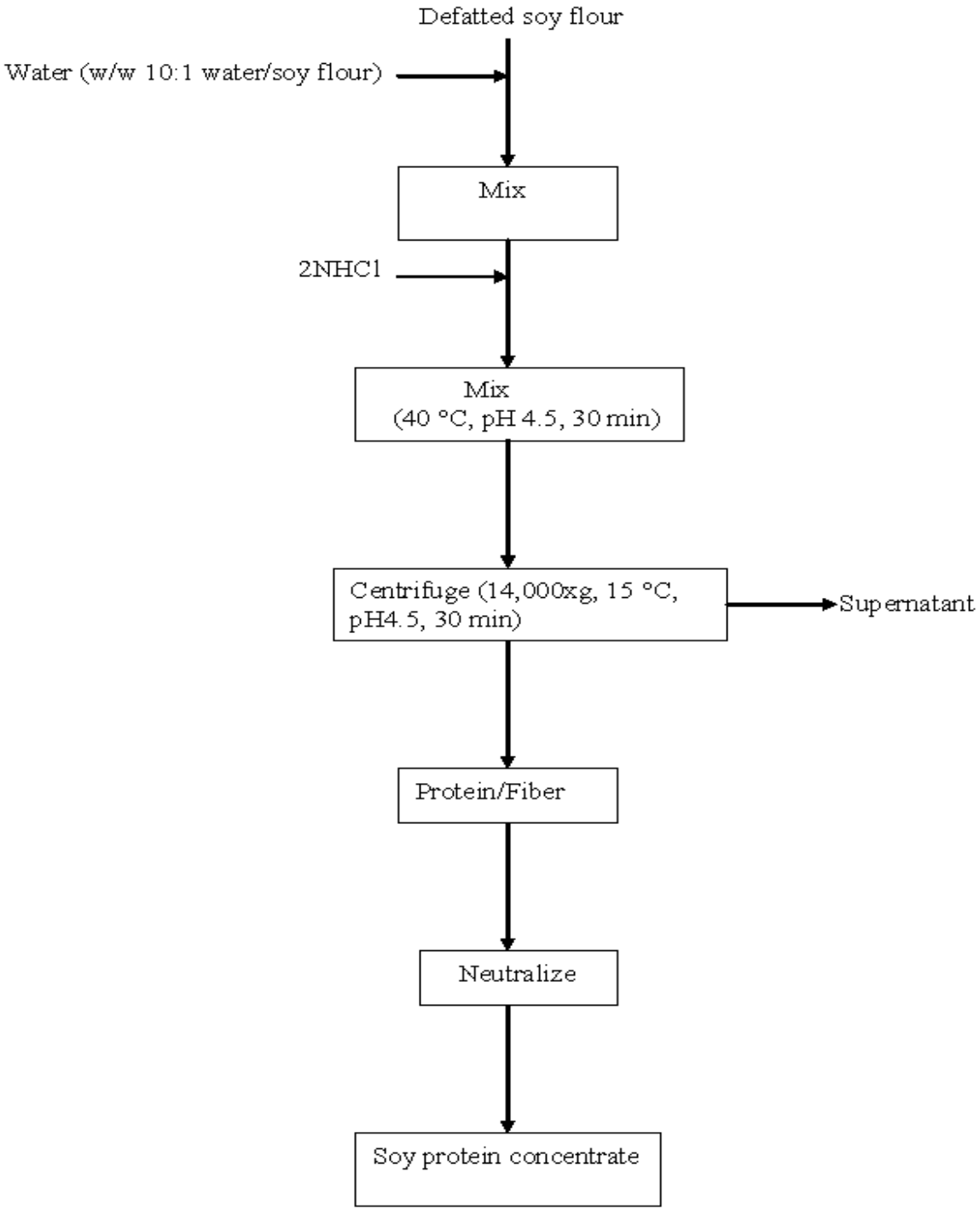


Figure 1.5. Flow chart for the production of soy protein concentrates (SPC) (Wang et al., 2004)

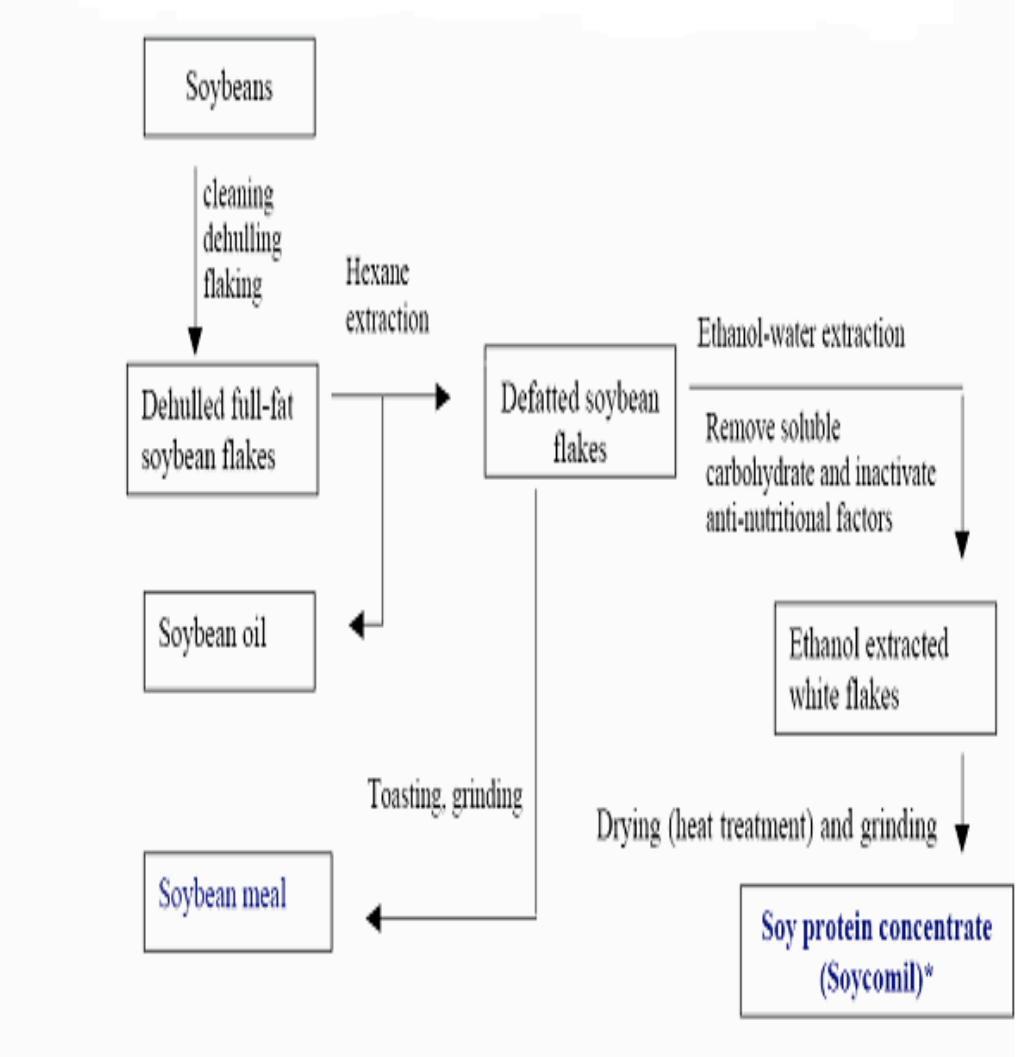


Figure 1.6 Procedure for producing SoyComil K (Archer Daniels Midland company, Netherlands "ADM" 2007) (ADM company 2006)

1.5.3 Soy protein isolates

Soy protein isolates (SPI) contain approximately 5% moisture, 91% protein, ~ 0% fat, ~ 0% carbohydrate, and 4% ash (Stauffer, 2002). Traditionally, SPI is obtained from defatted soy flakes or flour by extracting the soy flakes/flour using a dilute alkali (pH8-9) with subsequent centrifugation for the removal of the insoluble materials, producing soy protein, oligosaccharides and minerals. Acidification of the soy dispersion to pH 4.5 using a food grade acid (sulfuric acid, phosphoric acid or hydrochloric acid) causes the selective

recovery of the proteins due to their precipitation and concentration into a curd. Subsequent washing of the curd for the removal of non-protein solubles, neutralization (pH 7) and spray drying produces SPI as shown in Figure 1.7 (Alibhai *et al*., 2006 and L'hocine *et al.*, (2006).

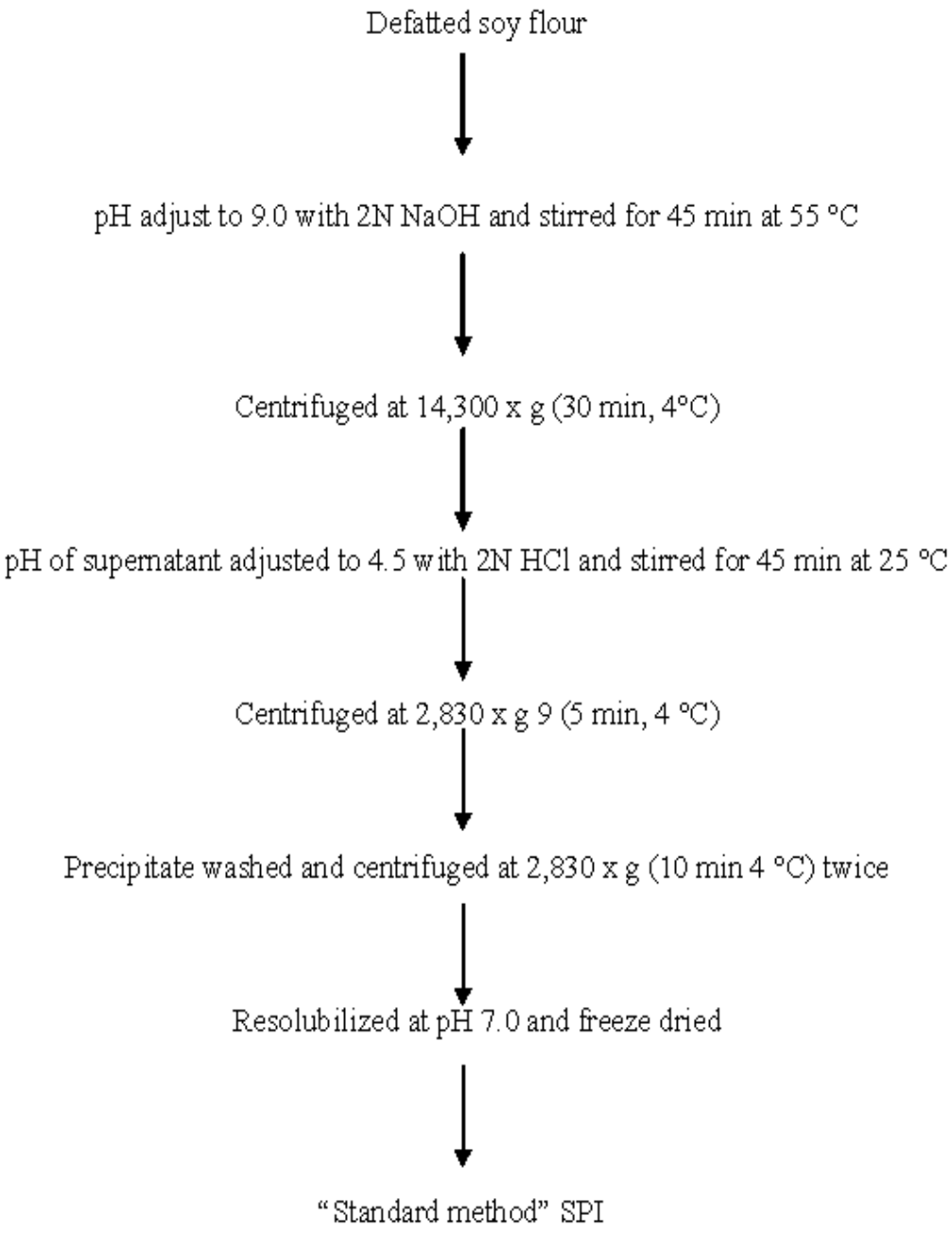


Figure 1.7 Flow chart for the production of soy protein isolates (SPI) (L'hocine *et al*., 2006).

1.6 Soy protein denaturation and associated molecular interactions

Soy protein structure can be modified by different treatments to improve specific functional properties. It has been demonstrated that the functionality depends on the degree of dissociation, denaturation, and aggregation of glycinin and β-conglycinin fractions (Sorgentini *et al*, 1995). The onset denaturation temperature β-conglycinin is 70°C, while that of glycinin is 80°C at neutral pH (Renkema and Van Vliet, 2002). Protein denaturation is any change of original native structure without alternation in sequence of amino acids (Adler-Nissen, 1976). Denaturation occurs because the bonding interactions responsible for the secondary structure and tertiary structure are disrupted; these include electrostatic, hydrophobic interactions and hydrogen bonding, covalent bonds and ionic bonds (Cramp, 2007). Protein denaturation can be measured by differential scanning calorimetry (DSC), which has been widely used to characterize the thermal properties of food proteins, including heat-induced denaturation (Tang *et al.*, 2006a).

The structure of a protein molecules or entities is determined by the type and strength of molecular forces that operate within it; such forces can be divided into two broad categories: intra- and inter-molecular forces. Intra-molecular forces operate within the fundamental units of a protein molecule (ionic interaction, hydrophobic interaction, hydrogen bonds, covalent bond, and Van der Waal forces). Inter-molecular forces operate between, rather than within, the protein molecules (protein- protein interactions) (Boonyaratanakornkit, 2002 and Kildahl, 2008).

1.6.1 Electrostatic bonds

Electrostatic interactions are known to play a crucial role in protein structure and function (Roy and Taraphder, 2007). Electrostatic interactions are collectively known as Van der Waals forces and include dipole-dipole interactions, London dispersion forces, and hydrogen-bonding. These forces are less than 1/6 as strong as covalent or ionic bonds (Cramp, 2007). Estimation of electrostatic bonds is the heart of any theoretical modeling of proteins. The electrostatic interaction between proteins or peptides can be reduced by addition of 0.3M NaCl; the counter- ions of NaCl interrupt electrostatic interactions leading to the breakdown of electrostatic bonds (Damianou and Kiosseoglou, 2006; Zhong *et al*., 2006).

Figure 1.8. London dispersion forces-induced dipoles (Cramp, 2007).

1.6.2 Ionic bonds

Ionic bonds are very strong and occur in proteins when salts are present. These are created by the attraction between the positive and negative charges on salts (Na^+, Cl^-, K^+, Ca^{2+}, and so on e.g. $Na^+ — Cl^-$). The charges created by salts can affect on functional properties of soy protein (Cramp, 2007).

1.6.3 Covalent bonds

Covalent linkages of amino acids in protein are largely limited to the peptide bonds. The most common exception to this rule is the disulfide bond, a sulfur- sullfur chemical bond that results from an oxidative process that links nonadjacent (in most cases) cysteines of a protein (Kadokura *et al*., 2003). Disulfide bonds are covalent bonds that may break and form under appropriate thermal conditions, such as the disulfide bonds between 11S acidic and basic subunits in soy protein (Figure 1.9) (Cramp, 2007). Identification of covalent (disulfide) bonds can be done by dispersing protein samples in solvent containing reagent of 0.2M 2-Mercaptoethanol, which reduces disulfide bonds to sulfhydryl groups (Zhong *et al*., 2006).

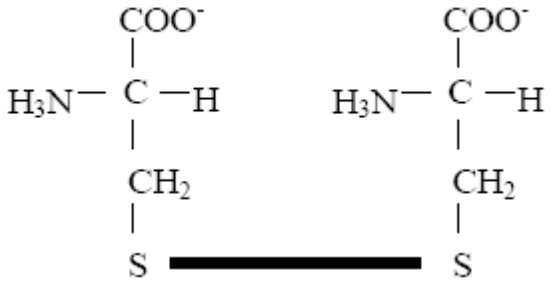


Figure 1.9 Covalent disulfide bonds between two cysteine residues (Cramp, 2007).

1.6.4 Hydrophobic bonds

Hydrophobicity is defined as the molecular driving force behind many important biological processes, such as protein folding (Li *et al*., 2007). Hydrophobic interactions are due to repulsion of water by hydrophobic molecules (Cramp, 2007). Identification of hydrophobic bonds can be done by dispersing protein samples in solvent containing 8M urea. Zou *et al.*, (1998) reported that urea binds to amide groups through hydrogen bonds, decreasing the hydrophobic effect through dehydration of protein molecules.

1.7 Properties of soy bean proteins in food systems

Thousands of tonnes of soy proteins are used world-wide as a functional ingredient in the food industry. The main functional properties of soy proteins are hydrating capacity, solubility, colloidal stability, gelation, emulsification, foaming and adhesion/cohesion. In addition it is applied as a fat substitute in meat, fish, milk, cereal-based products and infant formulation (Martins and Netto, 2006). Soy proteins in their various forms have functional properties that make them useful in food systems (Table 1.1).

Table 1.1. Functional properties of soybean properties in food systems (Wolf, 1970)

Functional property	Soy form	Food system
Emulsification	All forms	Cakes, potages, frankfurters, bologna, sausages and breads
Fat absorption	All forms	Bologna, frankfurters, sausages, and meat burgers
Stabilization	All forms	Frozen desserts, bologna, sausages, potages and frankfurters
Water absorption	Flours and concentrates	Confections, breads, cakes and macaroni.
Texture Viscosity Gelation	 All forms Isolates	 Potages, gravies and chilli. Imitation minced meats.
Chip and chunk formation	Flours	Imitation meats.
Fiber formation	Isolates	Imitation meats.
Dough formation	All forms	Baked goods.
Film formation	Isolates	Frankfurters and bologna.
Cohesion	Flours and concentrates	Baked goods, simulated meats and macaroni.
Adhesion	concentrates and isolates	Rolls, lunch meats, meat patties and sausages.
Elasticity	Isolates	Baked goods and simulated meats.
Color control Bleaching Browning Aeration	 Flours Flours Isolates	 Breads Breads, pancakes and waffles. Whipped toppings, chiffon mixes and confections.

1.7.1 Soy protein solubility

Soy protein solubility is probably its most important property in foods, not only because soy ingredients must form stable dispersions when incorporated into beverages and other food systems, but also because other functionalities, such as gelling, emulsifying and foaming, are closely associated with solubility (Wang and Johnson, 2001). Solubility is the amount of a solute that can be dissolved in solvent. Thermodynamically, the protein solubility is the protein concentration in the solvent in a sample or two-phase system in balance state. Mathematically, the solubility degree of a protein is the amount of protein present in liquid phase in relation to the total amount of protein in liquid and solid phases (Hall, 1996). The protein solubility also can be defined as a certain quantity of the protein retained in the supernatant after the dispersion has been centrifuged for a certain time period (Pelegrine and Gasparetto, 2005). In biological samples proteins are present in their native state. Solubility refers to proteins that are not aggregated or are present in aggregates too small to sediment upon centrifugation (Renkema *et al.,* 2000). Often proteins in their native state are not soluble, and should be denaturated to help solubilisation (Berklemn *et al*, 2004). Knowing the solubility profile of soy proteins in various environmental conditions is important to the industry in evaluating other physicochemical and functional properties in order to screen them for potential applications. Solubilities of soy products are highly dependent on the physicochemical states of protein molecules, which are either favourably or adversely affected by heating, drying, and other processing treatments during their manufacture and storage. This property is therefore one of the most widely used characteristics of protein products. The solubility characteristics of soy protein products can be influenced by a wide range of environmental conditions, including pH, ionic strength, and temperature (Lee *et al.*, 2003).

1.7.1.1 Effect of heat treatment on soy protein solubility

Thermal treatment is the oldest and most frequently used method for modification soy proteins. The purposes of the thermal modification of soy proteins are different. Thermal treatments reduce protease inhibitor activity, eliminate lipoxygenase and volatile compounds that induce undesirable flavours, and improve specific functional properties (Barac *et al.,* 2004). At higher temperatures some reactions may take place, such as breakdown of S-S bonds with release of H_2S, release of NH_3 from amide groups, dissociation of subunits and /or breakdown of these subunits into compounds of small

molecular weights, which may be responsible for the enhanced protein solubility (Figure1.10) (Shimada and Cheftel, 1988). Heating of soy protein probably causes dissociation of quaternary structure, releasing smaller peptides and facilitating their solubilisation (Yamagishi *et al*., 1983; Rangavajhyala et al., 1997).

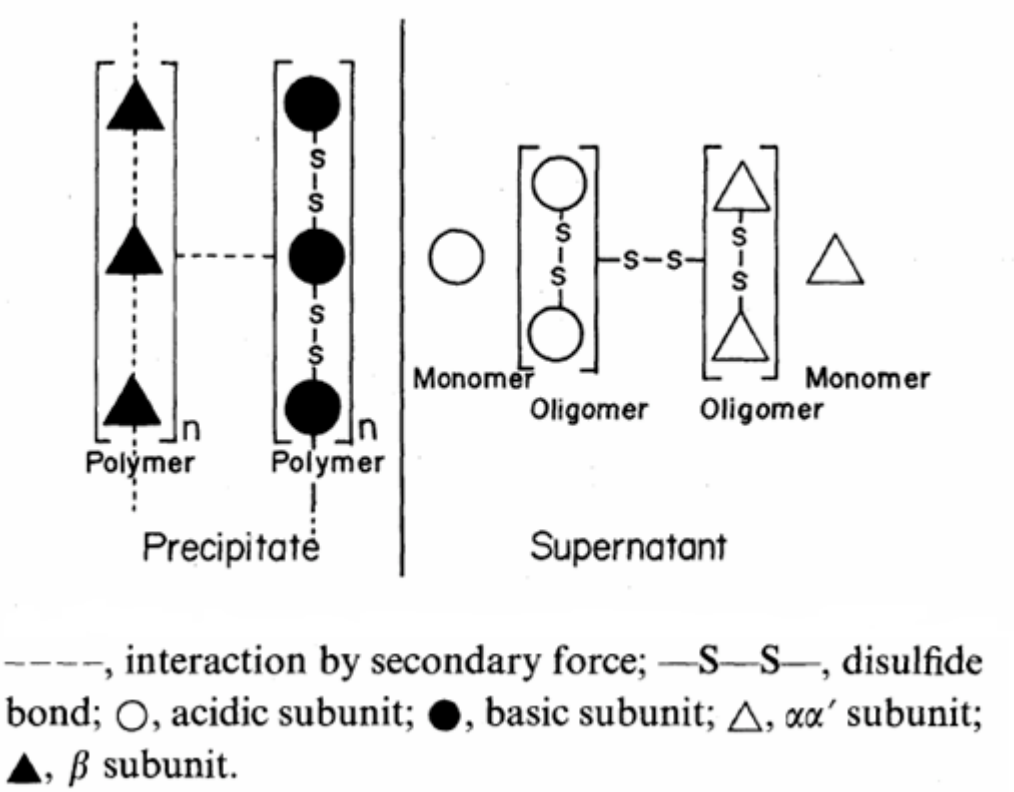


Figure 1.10 Schematic subunit interactions between 7S and 11S globulin on heating (Yamagishi et al., 1983)

1.7.1.2 Effect of pH on soy protein solubility

The pH of the solution affects the nature and the distribution of the protein's net charge. Generally, the proteins are more soluble in low (acid) or high (alkaline) pH values because of the excess of charges of the same sign, producing repulsive forces among the molecules and, consequently, contributing to its solubility (Pelegrine and Gasparetto, 2005). Protein usually has the least solubility at the isoelectric point (PI), here the electrostatic repulsive forces are insufficient to prevent extensive aggregation and less water interacts with the protein molecules (Malhotra and Coupland, 2004; Pelegrine and

Gasparetto, 2005). Molecular forces such as hydrophobic and electrostatic interactions can be manipulated by pH to produce proteins with varying structural conformations and hence functional properties such as solubility (Aluko and Yada, 1995). Under alkaline conditions, some hydrogen bonds in protein molecules are broken, causing it to assume a configuration somewhat more open than its original configuration, causing many buried peptide groups and side chains to become exposed to solvent (Mirsky and Pauling, 1936).

1.7.1.3 Effect of protein concentration on soy protein solubility

It is often difficult to increase protein concentration up to a high level without causing precipitation or aggregation (Golovanov *et al.*, 2004). Protein concentration has a significant effect on solubility, there is greater probability of protein-protein association if the protein concentration is high (Ranadheera, 2000). As the concentration of protein increases, its solubility decreases, probably caused by increased protein-protein interaction, causing an increase in the burial of charged groups (Pace *et al.*, 2004; Wagner *et al.*, 2000; and Sorgentini *et al*., 1995). When soy protein is at high concentration it will be aggregated, while at lower concentrations various types of soluble complexes may form (Roesch, and Corredig, 2005).

1.7.1.4 Effect of salt (NaCl) on soy protein solubility

Addition of salt to water alters the hydrogen bond length and the chemical structure of the liquid. Change in salt concentration produce a gradient in hydrogen bond strength as a function of salt concentration that varies from salt to salt (Ferreira-Machado et al., 2007). The effect of salt concentration on protein solubility depends on a salting-in region at low salt concentrations and a salting-out region at high ionic-strength solutions (Curtis *et al*., 1998). The increase of salt concentration reduces the solubility (salting out) resulting from competition between the protein and saline ions for binding to water molecules. Consequently, the water in the protein neighbourhood will be removed increasing the protein-protein interaction, thus leading to aggregation of the protein molecules, followed by precipitation (Ferreira Machado et al., 2007). At low salt concentration solubility of protein increase, due to salting in phenomenon by which saline ions interact with groups of opposite protein charges to form a double layer of ionic groups, thereby reducing the electrostatic interaction among the protein molecules and increasing their solubilisation (Hall, 1996).

1.7.1.5 Effect of sugars (monosaccharide, oligosaccharides and polysaccharides) on soy protein solubility

The significance of the sugar and protein interaction in food science are of fundamental importance to the quality of food products through their effect on properties of both aqueous phase and proteins in particular (Semenova *et al*., 2002). Sugars influence the heat induced denaturation of proteins, which can be divided in two categories. Firstly, sugars can act to stabilize proteins against heat denaturation by increasing the onset temperature of heat denaturation. The second effect of sugars on protein is covalent bonding of reducing sugars with available amino groups of amino acids protein through Maillard reaction (Semenova *et al*., 2002; Gu *et al.*, 2008), as illustrated in Figures 1. 11 and 1.12. The Maillard reaction was first observed by Louis Maillard in 1912, which results in formation of dark brown colors, flavors, and aromas. Non-enzymatic browning reactions are desirable in foods such as cookies and cakes. However, in other food systems there is a concern with the formation of carcinogens or the loss of the essential amino acid, lysine. Maillard reactions usually occur during processing and storage of foods (Morales and Van Boekel, 1998; Cramp, 2007).

The Maillard reaction is regulated by water activity, type of amino acids available, and type of sugars, oligosaccharides or polysaccharides present (Davies *et al,* 1998). Reducing sugars can consist of mono- or disaccharides that contain aldehyde or ketone reactive groups; Berg et al., 2007). Different sugars exhibit different rates of reactions and reactivity depending on how fast the ring opens (Cramp, 2007). The Maillard reaction could also occur with oligosaccharides or polysaccharides that contain reducing groups. Oligosaccharides are relatively low molecular weight short polymers of several monosaccharides (<20) joined by glycosidic bonds (Nelson, 2008), on the other hand, polysaccharides are high molecular weight polymers of monosaccharides (>20), resulting in branched polymeric structures; Berg et al., 2007). Examples of polysaccharides are starch, pectin, gums. Some polysaccharides contain one type of monosaccharide, this called homopolysaccharides (e.g., starch, cellulose and glycogen) and other contain more than one type of monomer are known as heteropolysaccharides (e.g., pectin, hemicellulose and gums) (Cui, 2005). Carbohydrates (oilgosaccharides and polysaccharides) can be hydrolysed into smaller fragments by enzymes (α-amylase, cellulose, hemicellulase, pectinase). Starch may be hydrolysed by amylase, pectin by pectinase, cellulose by

cellulose, and hemicelluose by hemicellulase (Kashyap *et al*., 2001; Kaya *et al*., 1996; Mussatto *et al*., 2008; and Tester and Sommerville, 2001).

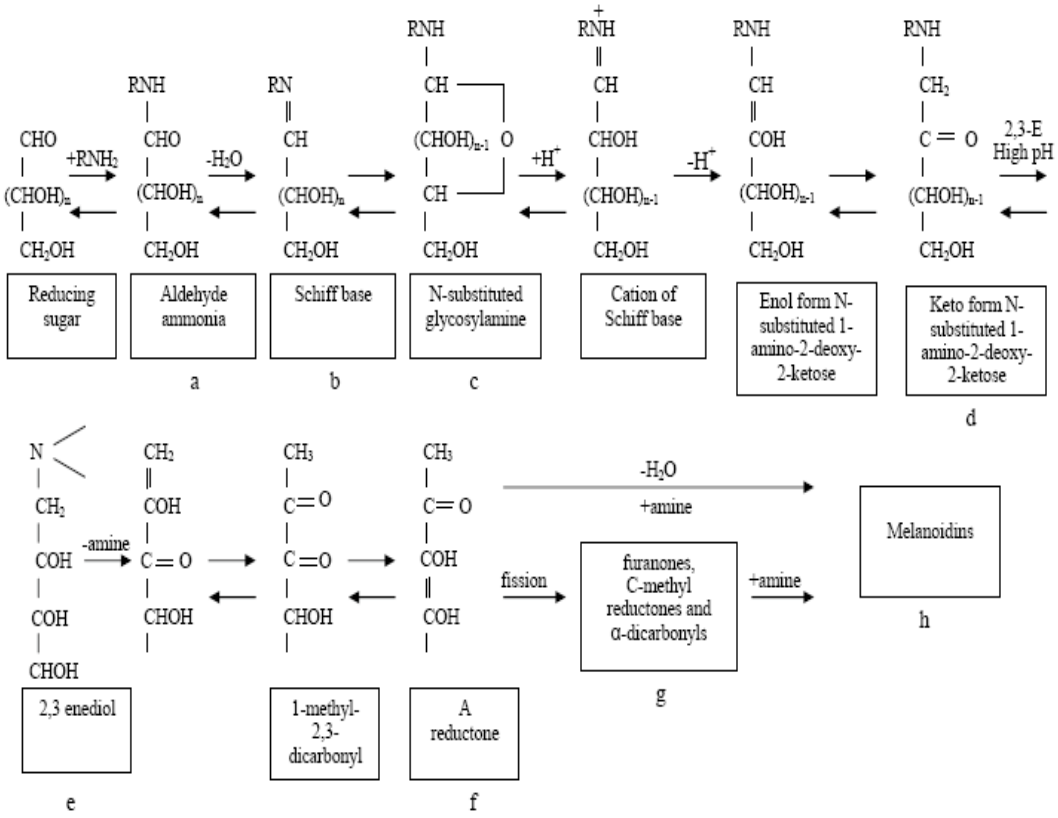


Figure 1.11 Maillard reactions for protein and reducing sugar combinations around pH 7, modified from Cramp (2007).

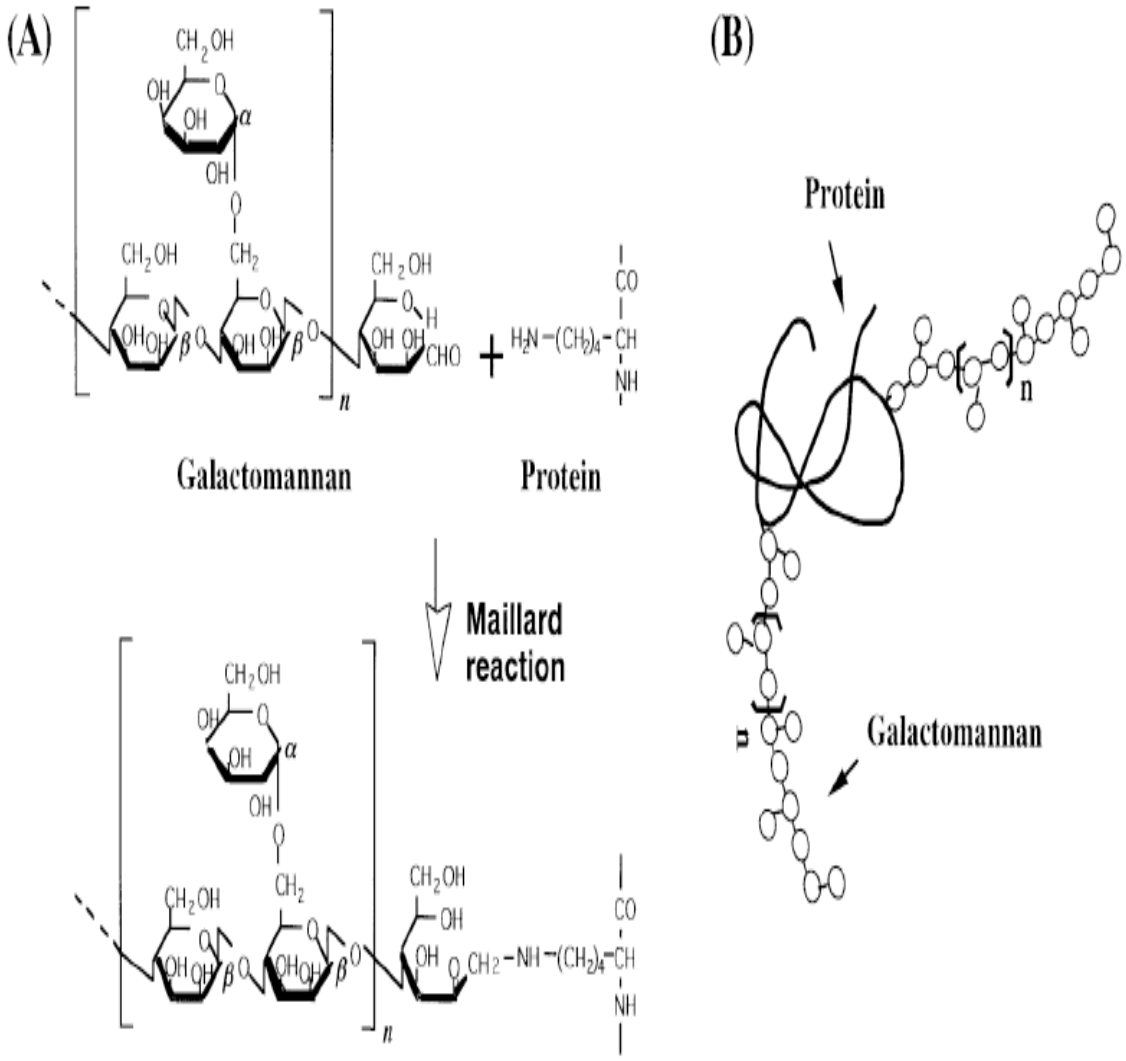


Figure 1.12 Schematic presentation of the binding mode of a polysaccharide with a protein through the Maillard reaction (A) and the resulting protein-polysaccharide conjugate (B) (Kato, 2002).

1.7.1.6 Effect of enzymes on soy protein solubility

The expanded use of enzymes to modify protein functional properties has great promise for the food industry. The major advantage of using enzymes compared to other agents include their specificity, their effectiveness at low concentration and, under mild conditions, their general safety, thus eliminating the necessity for removing them from

finished products. In addition, enzymatic hydrolysis of proteins does not reduce their nutritional value (Bernardi Don *et al.*, 1991). Enzymatic hydrolysis can be responsible for (1) a decrease in hydrolysate molecular weight (2) increase in ionisable group number, and (3) exposure of previously concealed hydrophobic groups (Lamsal *et al.*, 2007). Proteinase can increase solubility of soy protein by reduction of its secondary structure and to enzymatic release of smaller polypeptide units from protein (Kong *et al*., 2008). Treatment of soy protein concentrate with carbohydrate-hydrolysis enzymes (pectinase, cellulase and hemicellulase, α-amylase) can affect the solubility of soy protein (Abdel-Aziz *et al*., 1997). This is not surprising since soy protein concentrate contains approximately 23% carbohydrates consisting of a soluble (soluble starch and oligosaccharides, monosaccharides) and an insoluble fraction (pectin, cellulose, hemicellulose and insoluble starch and oligosaccharides; Blaney et al., 1996; Nakamura et al, 2001; Liu, 2004 and Peisker, 2001).

1.8 Hydrophobicity of soy protein

Hydrophobicity is usually understood as measuring the relative tendency of a protein to prefer a non-aqueous to an aqueous environment. It is also defined as a measure of the tendency of proteins to aggregate in solution. Both measures express some kind of phobia of the analytes towards the aqueous medium. Hydrophobicity is one the most important structure-related factors influencing functional properties of proteins and surface hydrophobicity is significantly correlated with protein gelation properties. The fluoresceient probe (with 1-anilino 8- naphthalene sulfonate “ANS”) method is widely adopted in researches to determine the surface hydrophobicity due to simplicity and high sensitivity. Wanger *et al.*, (2000) observed that the lower the solubility, the lower the surface hydrophobicity exposed by the proteins. This could be explained in two ways: (a) the protein species undergoing aggregation are more hydrophobic, so that only the hydrophilic ones remain soluble; and (b) as the proteins aggregate they hide or occlude the hydrophobic zones, leaving part of the proteins as soluble aggregates of low surface hydrophobicity. Hydrophobicity is a major factor that controls soy protein solubility. Nakai, (1983) observed an increase in the hydrophobicity upon heating of proteins, indicating unfolding of the molecules.

1.9 Water holding capacity

Water holding capacity (WHC) is a quantitative indication of amount of water retained within a protein mixture under defined conditions (Huang and Kinsella, 1986). Kneifel *et al.,* (1991) defined it as the ability of a food structure to prevent water from being released from the three-dimensional structure. Generally the water held in a protein structure can be divided into two types: 1) the part bound to the molecule that is no longer available as a solvent and 2) the other part trapped in the protein matrix or a corresponding co-matrix (polysaccharide, fat). The first type can be regarded as absorbed water and the second as retained water. In most cases, the water-holding capacity of a protein matrix is determined by both the amount of absorbed and retained water. The absorbed water, which is more tightly bound to the protein molecules, will be considered first. This type of water is largely influenced by the physicochemical parameters that directly affect the proteins and the surface properties of the protein molecules that interact with the dissolving solution. This means that the water holding capacity depends not only on pore and capillary size but also on the charges of the protein molecules (hydrophobic interactions, hydrogen bonds, S-S bonds, acids, bases) as well as on Van der Waals' forces (Kneifel *et al.,* 1991). Water holding capacity is influenced by several parameters such as pH, temperature, protein size, shape, lipid and carbohydrate associated with protein, the hydrophilic-hydrophobic balance of amino acids and the presence or absence of surfactant (Han and Khan, 1990). Addition of polysaccharides to protein could increase water holding capacity through gelation by increasing the gel network, with smaller pores and greater capillary forces (Hua *et al.*, 2003; Maltais *et al*., 2005; Yamamoto and Cunha, 2007), and also by their ability to bind water with their hydroxyl groups (Uresti *et al*., 2003). Glycation of SPI increased its water holding capacity, due to increase in net charge via Maillard reaction (Gu *et al*., 2008). This could also have been due to higher concentration of high molecular weight polymers taking part in the gel net work and resulting in better entrapment of water in the glycated protein gels (Gu *et al*., 2008).

1.10 Emulsifying ability of soy protein

Commercial preparations of soy protein may cause physical and chemical changes that in turn affect the proteins functional properties. Consequently, different commercial soy varies widely in their emulsifying properties reflecting their difference in composition, conformation, net charge and structure. However, many factors in the surrounding

environment such as pH, ionic strength, temperature and presence of other components affect functional behaviour, making it very difficult to predict soy protein emulsifying ability in a given system (Elizalde *et al*., 1996).

1.10.1 Properties of soy protein emulsions

An emulsion is formed from one immiscible phase distributed as small droplets in the matrix of a second phase by means of an emulsifying agent. The dispersed phase is the discontinuous phase and the dispersion medium is the continuous phase. Emulsions are characterized by the presence of at least one polar hydrophilic liquid and at least one lipophilic liquid (Al-Malah *et al*., 2000). The two basic types of emulsions are dispersions of a lipophilic or oil phase in a hydrophilic or watery phase or versa. With oil and water being the most common liquids for the preparation of food emulsions, these basic types of emulsions are referred to as oil-in-water (O/W) emulsions and water-in-oil (W/O) emulsions, respectively. More complex types consist of three or more phases, which can be achieved by e.g. dispersing a W/O-emulsion into a second watery phase, leading to a water-in-oil-in-water (W/O/W) (Schubert *et al.*, 2006). The basic types of emulsions are depicted in Figure 1.13. Emulsions are characterized by having a large interfacial area between the liquid phases, allowing a faster exchange process or chemical reaction to take place at the interface. Emulsions form the basic structure of many foods, pharmaceuticals, cosmetics, laundry and cleaning agents and lubricants (Al-Malah *et al.,* 2000). Emulsions uses can be classified into two different types. Some emulsions are end products which should remain stable towards creaming and coalescence during their production and shelf-life such as mayonnaise and salad dressings. Emulsion can also be used as ingredients, which participate in the formation of more complex products such as yogurts, gelled systems, and ice creams. The behaviour of food emulsions is determined by the three phases of the system: the fat or oil phase, the interfacial phase and the aqueous phase.

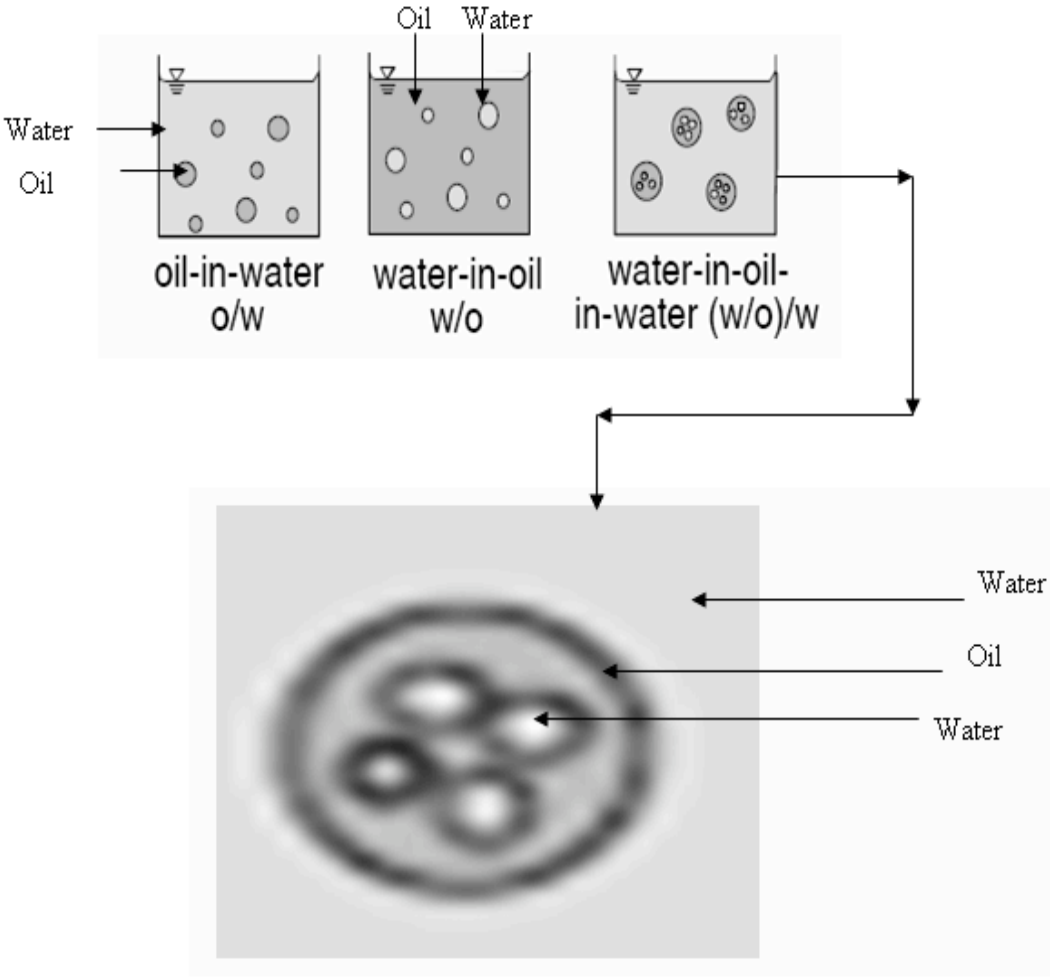


Figure 1.13 Principle types of emulsions (Schubert et al., 2006)

The oil may be partly or totally crystallized depending on temperature. The interface may be composed of proteins or of small emulsifiers such as monoglycerides, phospholipids, fatty acids or mixtures of these surface-active molecules. The aqueous phase may contain ions and biomolecules such as polysaccharides or proteins, which may exert stabilizing or destabilizing effects (Leal-Calderon *et al*., 2007). Food emulsion properties depend on several factors such as temperature, homogenization pressure, food compositions, type and concentration and of emulsifier and/or stabilizer (Peamprasart and Chiewchan, 2006).

1.10.2 Mechanism of emulsion formation

Globular proteins such as soy and whey protein are used as emulsifiers in number of commercial products because of their ability to facilitate emulsion formation and enhance long-term emulsion stability. Globular proteins rapidly adsorb to an oil-water interface formed when an aqueous phase and oil phase are homogenized where they unfold and facilitate droplet disruption because of the decrease in interfacial tension. These conformational changes promote hydrophobic and electrostatic interactions, which cause stabilization of viscoelastic protein film around oil droplets.

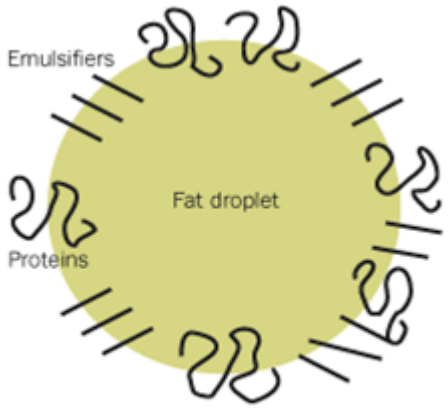


Figure 1.14 Surface-coated oil globule (Raikos, 2006)

Emulsifying proteins usually contain α-helical or random coiled structures in aqueous solution and may lose or gain α-helical structure through interfacial adsorption (Annan, *et al*., 2006)**.** They also help to prevent droplet coalescence within the homogenizer due to the formation of a protective coating around the droplets. Adsorbed proteins can also improve the long-term stability of oil-in-water emulsions by preventing droplet aggregation by generating repulsive colloidal interactions between the droplets e.g., electrostatic and steric repulsion (Chanasattru *et al*., 2007).

1.10.3 Emulsion stability

The formation of a food emulsion is dynamic, yet thermodynamically unfavourable, process due to the increase in interfacial area following emulsification. Hence, after enough time, any emulsion will collapse as the two phases attempt to minimize contact area. There are five main mechanisms, which can contribute to emulsion instability: (1) creaming; (2) flocculation; (3) Ostwald ripening; (4) (partial) coalescence; and (5) phase inversion. Creaming (or settling) is due to density differences between two phases under the influence of gravity, which leads to phase separation. Flocculation is best described as the aggregation of particles due to weak attractive forces between colloids (Figure1. 15). Ostwald ripening is the growth of larger droplets at the expense of smaller ones and is related to the solubility gradient found between small and large droplets. Partial coalescence is two colliding droplets which form single large droplet (Figure 1.15). Phase inversion is where an oil-in-water (O/W) emulsion becomes water-in-oil (W/O) emulsion, as during the churning of butter (Rousseau, 2000).

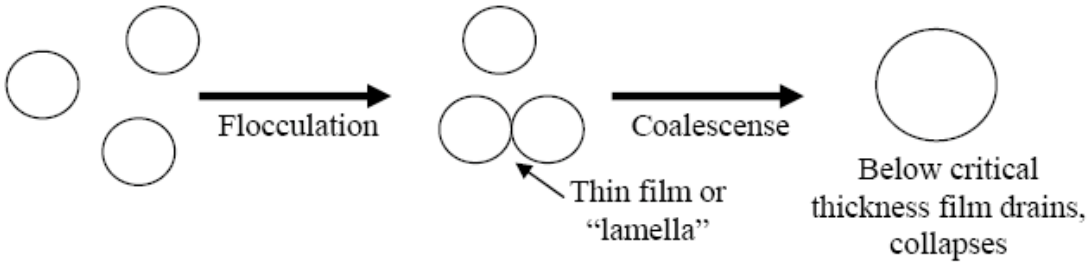


Figure 1.15 Schematic presentations of flocculation and coalescence (Cramp, 2007)

1.10.4 Factors that affect protein-stabilised emulsion properties

1.10.4.1 Effect of heat treatment

Heating an emulsion usually causes a reduction in the interfacial tension between the oil and water phase, which would be expected to facilitate the production of small droplets (Floury *et al.,* 2000). In addition, heat treatment affects viscosity. The viscosity of both oil and aqueous phase is temperature-dependent and decreases with increasing temperature, which is expected to facilitate the production of small droplet. On the other

hand, temperature affects the rearrangement of the fat globules, which certainly would modify the rheological properties of an emulsion (Peamprasart and Cheiewchan, 2006).

1.10.4.2 Effect of high-pressure homogenization

The most common applications of conventional homogenization are to form stable fine emulsions and to disperse, mix and process colloidal products. The two major mechanisms initiated during homogenization are intense turbulence and shear flow fields; turbulence being the most predominant force in the emulsification process, which disrupts the dispersed phase into small particles. Smaller fat droplets can be produced by increasing the turbulence (higher homogenization pressure) and larger fat droplets can be produced by reducing the turbulence (low homogenization pressure). Reducing the droplets size will improve the shelf life of the products by reducing the creaming rate and by increasing the surface area of the emulsifier to improve the coating ability (Gerung, 2005).

1.10.4.3 Effect of sugars (monosaccharide, oligosaccharides and polysaccharides in aqueous phase

Many food products contain both oligosaccharides, polysaccharides and proteins. In particular, mixtures of proteins and polysaccharides can be found among the ingredients of a wide range of colloidal food systems such yogurts, mayonnaise and ice cream (Neirynck et al., 2007b). A binary mixture of protein and hydrocolloid (polysaccharides) in aqueous solution can exhibit one of the three different equilibrium situation: (a) miscibility, (b) thermodynamic incompatibility or (c) complex coacervation (or complexation), thermodynamic incompatibility implies the separation in to two distinct aqueous phases, one rich in protein and the other rich in hydrocolloid. An incompatibility may lead to phase separation and destabilization of emulsions. Thus, protein-polysaccharide interactions should be well known in order to formulate ingredients in a more accurate way (Ercelebi and Ibanoglu, 2007). Proteins are present primarily as emulsion forming and stabilizing agents, whereas polysaccharides are mainly used as thickening and water-holding agents. In addition, both kinds of biopolymers may contribute to the structural and textural characteristics of food products through their aggregation and gelling behaviour (Neirynck *et al.,* 2007a). Polysaccharides can form weak electrostatic complexes with protein. They can induce depletion flocculation or in some cases, they are weakly adsorbing and can cause bridging flocculation. Interactions of this type lead to changes in emulsion stability,

and this will almost certainly alter the way in which SPI emulsion respond to heat treatment (Euston, *et al*., 2000).

Addition of polysaccharides to SPI emulsions enhanced the rate of emulsion droplet aggregation during heating, due to depletion flocculation, where emulsion droplets become aggregated by attachment of adsorbing macromolecules to more than one droplet at a time (Dickinson, 1992; Ye and Singh, 2006). Addition of glucose to soy protein containing emulsions produced emulsion with small droplet size. This was probably due to hydroxyl groups present in sugar units, which contribute to protein-sugar interactions in aqueous solution. These newly formed dipole-dipole interactions could either form a hydrophilic layer around the protein unit and, therefore increase the dispersibility of protein through protein hydration and/or alter the intermolecular interactions in such a way that folding and even dissociation may be formed (Baier and Mcclements, 2005; Ryan and Brewer, 2005).

1.11 Gelation properties of soy protein

Protein gelation is applied in many industrial food applications. In food products, it lends desirable sensory and textural properties. A gel can be defined as steady state flow of continuous network of macroscopic dimensions in a liquid medium (Oakenfull *et al*., 1997). Protein gel is a continuous network formed by denaturation of protein to a certain degree (Alting, 2003). There are three characteristics which constitute a gelatinous state. First, there must be at least two components in the system – a dispersing phase and a dispersed phase. Secondly, both the dispersion solvent and the dispersed component must be equally distributed throughout the gel system. Thirdly, the system must display specific rheological properties typical of a solid (Ranadheera, 2000). Gel forms depend on the extent of crosslinking of the initial molecules present, and this in turn is influenced by environmental conditions, concentration, temperature, heating time, and pH (Mcklem, 2002). Gel networks can be divided structurally to fine-stranded and coarse aggregated networks (Figure 1.16) depending on microscopic observations. A fine stranded network is characterized by transparent fibers that are a few times larger than protein molecules. A coarse network is characterized by non-transparent fibers with thickness 100-1000 times larger than protein molecules. A combination of network types (fine-stranded and coarse) could exist. The type of network formation depends on the environmental conditions during gel formation. As the pH approaches the isoelectric point, the network tends to becomes coarse, as well as when the ionic strength is increased (Renkema, 2001).

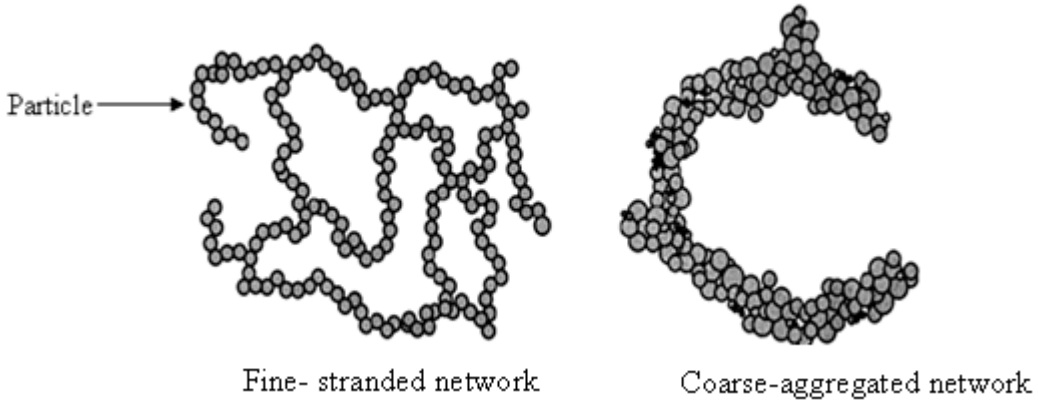


Figure 1.16 A Schematic diagram of gel network (Renkema, 2001)

1.11.1 Factors affecting gel formation

1.11.1.1 Heat treatment

The use of heat to denature globular proteins is the most common technique for forming gel networks (Ranadheera, 2000) and is necessary for gelation (Renkema, 2001). Heat induced gelation of protein can be performed in three steps. The first is a change in the protein system caused by heat, increasing intermolecular interactions owing to the partial unfolding of protein molecules. The second step is the aggregation of protein molecules by sulfhydryl-disulphide interchange and sulfhydryl oxidation within the preformed aggregates and perhaps between the aggregates to form the gel network. This occurs when the attractive forces between the molecules are sufficiently strong to overcome the repulsive forces. The third step is the occurrence of multiple hydrogen bonding that takes place on cooling (Christ *et al*., 2005).

Native protein → Denatured protein → Soluble aggregate → Gel network

1.11.1. 2 Protein concentration

Protein concentration plays an important role in formation and characteristics of a gel. There is certain concentration, which varies depending to protein utilization, below which gelation will not occur. As protein concentration increases, the strength and texture of gel increase (Mangino, 1984). The SH groups decrease markedly as soy protein concentration increase, since the oxidation of SH group to S-S bonds is favored at high protein concentration due to increased protein-protein interaction(Shimada and Cheftel, 1988 and Sorgentini *et al.*, 1995).

1.11.1.3 Effect of pH

The pH can have a marked effect on structure of soy protein and water holding capacity of protein and gel properties. The pH affects maintenance of the gel network through charge repulsion (Mangino, 1984). At pH >6 soy protein gels have low stiffness, while high stiffness occurs at pH < 6, due to the difference in association/dissociation behaviour of proteins (Renkema, 2001). Generally at a pH value far from the isolelectric pH (PI), the gel becomes transparent with fine-stranded, random aggregates,, while at pH close to the PI, the gel becomes coarse and thick stranded. (Alting, 2003).

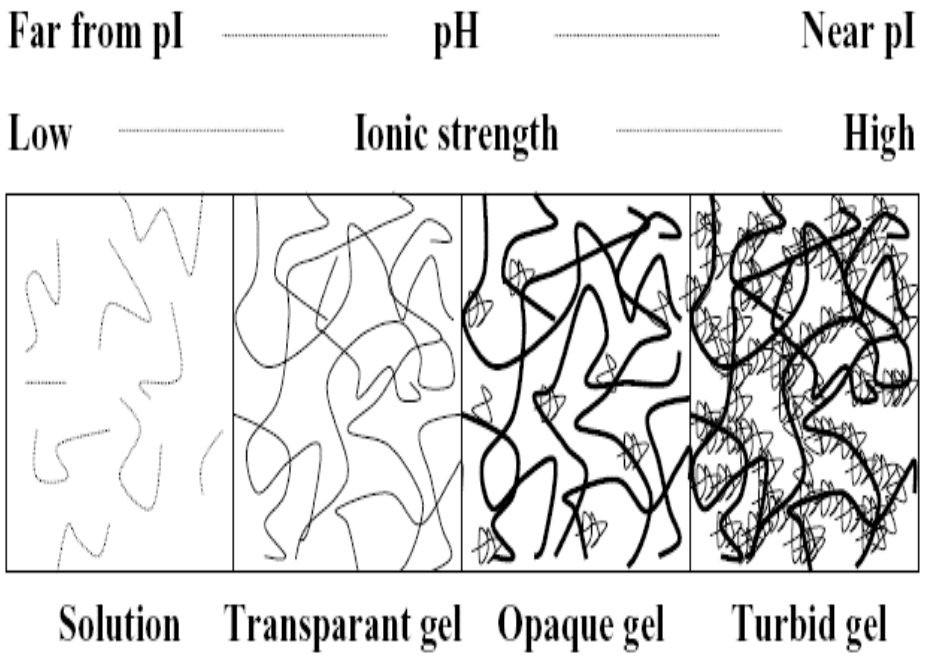


Figure 1.17 Influence of pH and ionic strength on final whey protein gel properties (Alting , 2003)

1.11.1.4 Effect of ionic strength (NaCl) on gelation

Addition of salt can enhance gelation of soy or whey protein, by denaturation and aggregation. At room temperature (22 ± 3°C) addition of small amounts of salt can produce a fine-strand transparent gel. However addition of large amounts of salt produce turbid particulate gel (Alting, 2003).

1.11.1.5 Effect of sugars (oligosaccharides and polysaccharides) on gelation

The addition of sugars increases the hardness of soy or whey protein gel, which could be attributed to the ability of sugars to form hydrogen bonds, increasing the number and stabilization of junction zones (Sabadini *et al.*, 2006). They would also interact with the protein and this interaction would have a synergistic effect on gel strength (Baeza *et al.,* 2002). This effect would be strongly related to Maillard cross-linking which might increase the molecular entanglement in the gel structure, preventing the rupture of weak non-covalent interaction, and thus stabilize gel network (Sun *et al.,* 2006).

1.11.2. Gelation properties of soy protein

It has been reported that gelling properties of soy protein depends on glycinin and conglycinin content, which are affected by such structural factors as subunit composition, accessibility and/or reactivity of –SH groups and hydrophobicity. It has also been demonstrated that heat-induced gelation of aqueous dispersion of soy globulins is affected by protein concentration, and time and temperature of heating (Kang *et al.*, 1991).

These proteins have complex quaternary structures that easily undergo association-dissociation reaction, depending on environmental conditions (Hermansson, 1986). Heating of soy proteins above 60°C is necessary to induce dissociation of the quaternary structure of globulins and to cause unfolding of the protein subunits and a consequential increase in viscosity. It has been demonstrated that glycinin gels are firmer and more elastic than *β*-conglycinin gels.

1.11.3 Texture analysis of protein gels

Texture profile analysis is now the most frequently used imitative test, which refers to the flow, deformation, and disintegration of sample under force. Texture relates to soild foods, and viscosity-the tendency to resist flow-related to fluid foods, texture parameters such as hardness, cohesion strength, and adhesiveness (Tunick, 2000). There are many factors, which should be considered in evaluating gel texture (Table 1.2). These are fracturability, hardness, adhesiveness, cohesiveness, springiness, gumminess, chewiness (Wilcke et al., 1979).

Table 1.2 Definitions of evaluating factor of food texture

Mechanical property	Definition	Reference
Fracturability (originally called brittleness)	with which the sample crumbles cracks or shatters. Not all samples fracture	Raikos, 2006
Hardness	The force required to deform a product to given distance	Mioche and Peyron, 1995
Cohesiveness	Determines how much of a gel structure is destroyed after compression as related to force	Raikos, 2006
Adhesiveness	The work required to overcome the attractive forces between the food surface and surface of other material which come in contact with food such as the plate, tongue, and teeth	Ju and Kilara, 1998
Springiness	How well a product physically springs back after it has been deformed during the first compression	Raikos, 2006
Gumminess	Energy required to disintegrate a semi-solid food to a state ready for swallowing. It only applies to semi-solid samples	Raikos, 2006
Chewiness	Number of chews needed to masticate a sample to a consistency suitable for swallowing.	Raikos, 2006

1.12 Effect of glycation of proteins on protein functionality in general

Many efforts have been made to improve the functions of food proteins by conjugation of polysaccharides through Maillard reaction in the dry state, nevertheless data on conjugation of protein with polysaccharides via Maillard reaction in liquid state are

scare. For example, the conjugation of hen egg lysozyme with dextran, galactomannan, or xyloglucan in the dry state was effective to improve the emulsifying activity of lysozyme and it was found that the conjugated lysozyme had new antimicrobial characteristics (Mu et al., 2006). The Maillard reaction can have a positive impact on the solubility of heat-denaturated globular proteins, this may be due to the fact that protein molecules become less hydrophobic after covalent modification with sugar molecules. This means that more polar groups are present on the surface area of the protein backbone, resulting in enhanced solubility in aqueous environment (Campbell *et al*., 2003). The Maillard reaction can also cause a pH reduction in protein gels due to the production of acidic side products. Decreased pH can cause changes in the gelation process, thus causing changes in the rheological properties of gels (Rich and Foegeding, 2000).

1.13 Yogurt

In this study, the modified soy proteins were applied in a yoghurt system. The section below gives an overview of yogurt manufacture.

Yogurts come in many forms (e.g. liquid, set and stirred curd), with varying fat contents (e.g. regular fat, low fat and fat-free) and flavours (e.g. natural, fruit, cereal, chocolate), and can be consumed in different ways (snack or part of a meal, as a sweet or savoury food). Yogurt's nutritional profile has a similar composition to the milk from which it is made but will vary somewhat if fruit, cereal or other components are added. Yogurts can be used as dietary supplements for infant consumption. Therefore, they cross the line between dietary supplements and healthy foods (Ganesh, 2006). The average composition of yogurt is 4-6% protein, 7-10% carbohydrate, 1-3% fat and the rest is moisture (Early, 1998; Yazici and Akgun, 2004). Traditionally, yogurt is a food produced by culturing milk or reduced fat milk using a mixed culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The milk is incubated with culture at 43°C until the pH reaches 4.5, then the yogurt is stored at 5 $\pm$ °C; however, this can vary from country to country (Guggisberg *et al*., 2007). The addition of organic acids (ascorbic, acetic, lactic, citric and phosphoric) or glucono-δ-lactone (GDL) can result in the formation of coagulum at pH < 4.6.

The main objections to the use of soybean products for yoghurt production are associated with beany flavour and the phenomenon of flatulence (i.e. production of carbon dioxide, hydrogen and methane by the intestinal flora during the breakdown and/or metabolism of the oligosaccharides raffinose and stachyose present in the soybean). It is possible that

these problems can, of course, be overcome by various processing techniques such as removal of unwanted fibers from soy protein (SPC) or SPI. New commercial grades of SPI are also distinctly neutral in flavour. It was therefore of interest in this study to develop a yoghurt with SPI, having improved texture and taste than commercially available soy yoghurt manufactured with soy milk.

1.14 Aims and objectives of this study

The rising cost of food-grade proteins from animal source, and increased food shortage in the world has increased the pressure on food scientists to develop new protein products, particular from vegetable proteins. Soy proteins have been used in a variety of food applications for many years. Some of the reasons for their use include their relatively low cost and availability compared to other competing food ingredients. The primary reason for the use of soy proteins, however, is their wide range of functional properties that help to stabilize food systems as well as provide sensory properties, such as texture, that consumers demand. The objective of this thesis was to study the effect of heat treatment and glycation in solution on five industrially important functional properties of soy protein, namely solubility, emulsifying ability, water holding ability, acid gelation ability in soy yoghurts and the heat stability of whey/soy protein emulsions. These objectives were achieved completing three tasks:

Task 1 Functional properties of SoyComil K

SoyComil K is an insoluble commercial SPC sold as animal feed. The present study aims to improve its functional properties such as solubility and emulsification to facilitate its use in industrial applications. Physical treatments (temperature, pH, and concentration), chemical treatments (NaCl and glycation with sugars) and enzymatic modification (carbohydrate hydrolysis enzymes and proteinase K) were used to effect changes in the solubility of the SPC.

Task 2 Soy protein isolate and polysaccharides in model yogurt

There is a consumer demand for imitation-dairy products made from non-dairy ingredients. Soy protein can be used to make dairy subsitutes, but there are a number of barriers to their use. These include the “beany” taste and difficulties reproducing the texture of milk protein based dairy products. In this task attempts were made to produce soy yogurt with high quality (smooth, homogenous texture and high water holding capacity (WHC)) using SPI. In addition to SPI, the yogurts were also made with different

sugars (glucose, pectin, amylopectin, starch, carrageenan, xanthan, guar gum and locust bean), and by different processing conditions (heat treatment, and homogenization) to further improve the texture.

Task 3 Destabilization of SPI emulsions

Proteins are valued by the food manufacturer, for their ability to emulsify oil and stabilize it in fluid emulsions. A barrier to the use of globular proteins such as whey and soy protein as emulsion stabilisers is their tendency to denature and aggregate when heated, which leads to coalescence of oil droplets and subsequent oil separation. Understanding the mechanisms of heat-induced breakdown of SPI emulsions will enable the food manufacturer to make better use of them in food applications.

In this task, heat-induced destabilization of SPI and Whey protein concentrate (WPC) emulsions was studied in two parts:

Part 1: A study of the rate of aggregation of SPI and WPC stabilized emulsions at different SPI concentrations and temperatures at pH 4.5. In addition, studied the heat stability of mixed WPC and SPI emulsions was done.

Part 2: A study of the effect different polysaccharides (pectin, carrageenan and xanthan) on heat stability of emulsion made with SPI at pH4.5.

Chapter Two

Materials and Methods

2 Materials and methods

2.1 Materials

SoyComil K (which is an insoluble kind of soy protein concentrate,72% protein, sold as animal feed), soy Arcon ® SJ (which is soluble kind of soy protein concentrate , 70% protein) and soy protein isolates (90% protein) were obtained from ADM (*Archer Daniels Midland company , Netherlands*). Whey protein concentrate was obtained from Arla Foods Ingredients amba Denmark. The commercial enzymes (α-amylase, proteinase K, cellulose, and hemicellulase), sugars (sucrose, glucose, lactose, ribose), starch, locust bean gum, carrageenan, guar gum, xanthan gum, amylopectin, and pectin), NaCl, glycine, DNTB, EDTA, urea, SDS, 2-mercaptoethanol, 1-anilinonaphthalene-8-sulphonate (ANS), phosphoric acid, glucono-δ-lactone (GDL), Coomassie Brilliant Blue G-250, Tris, acetic acid and 95% ethanol were obtained from Sigma-Aldrich Company in UK. Electrophoresis and electrophoresis materials were obtained from Invitrogen Co, UK. Sunflower oil was purchased from ASDA supermarket in Edinburgh, UK.

2.2 Methods

2.2.1 Preparation of soy protein concentrates (SPC)

SPC was isolated from defatted soy flour according to Wang *et al.*, (2004), with some modification (centrifuge speed and freez-dried).

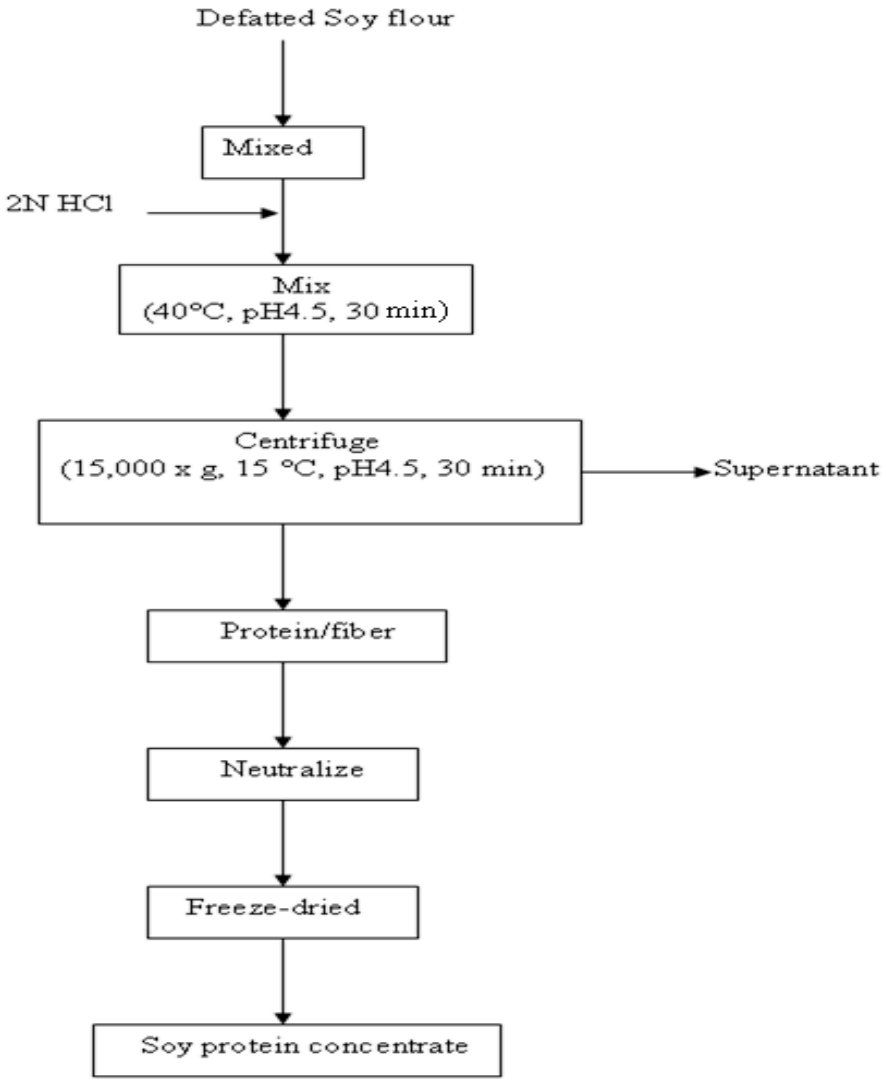


Figure 2.1 Laboratory preparation of soy protein concentrates (SPC)

2.2.2 Preparation of soy protein isolates (SPI)

SPI was isolated from defatted soy flour according to L'hocine *et al.*, (2006), with some modification (centrifuge speed).

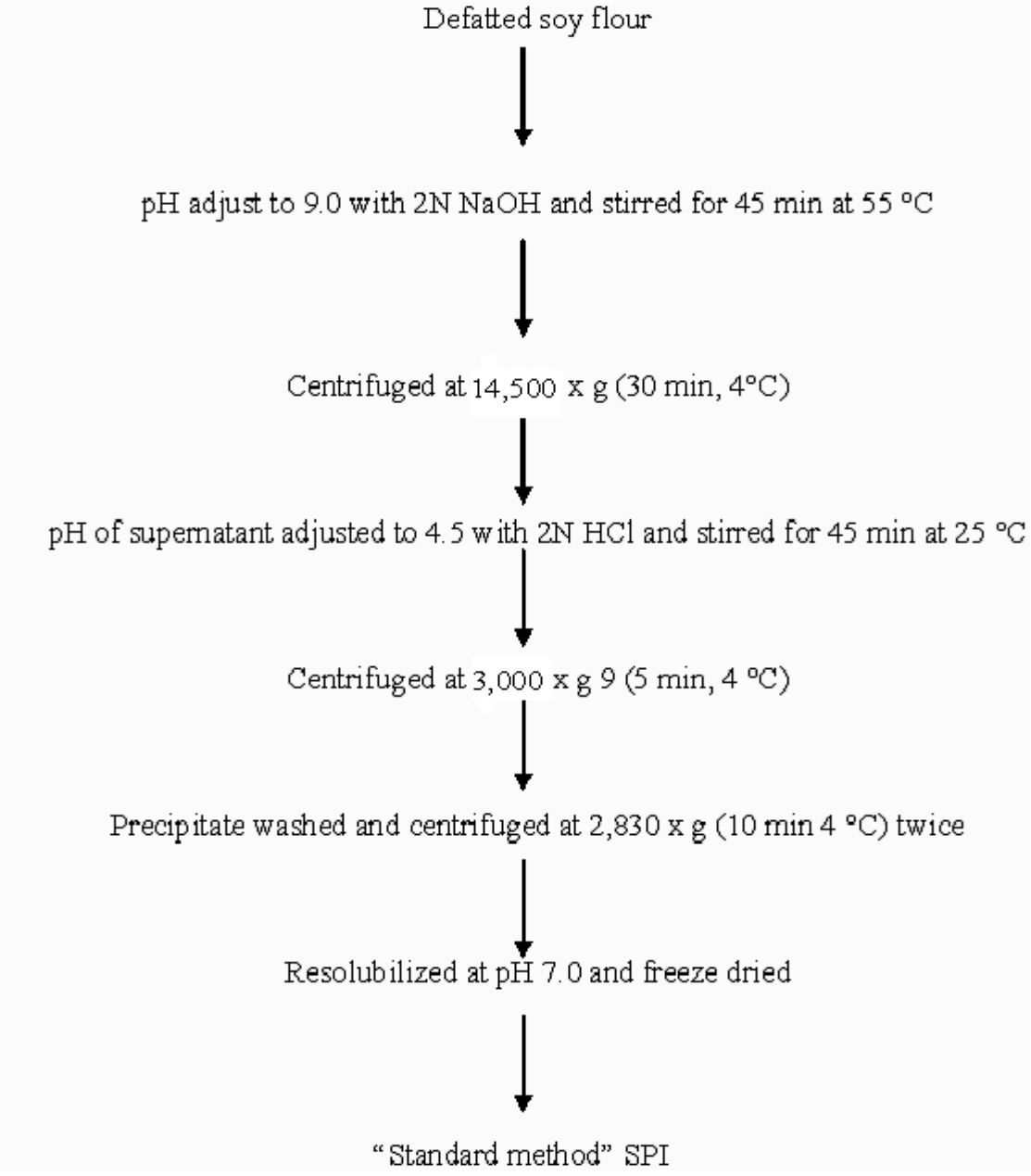


Figure 2.2 Laboratory preparation of soy protein isolates (SPI)

2.2.3 Preparation of emulsions

2.2.3.1 SoyComil K emulsions

Emulsions were prepared from unheated and heated (100°C for 10 min) SoyComil K (6% w/v) dipersions prepared as shown in Figure 2.3. The protein concentration was adjusted by adding the appropriate amount of water. The pH of the suspensions was adjusted to pH 7, following the treatments and diluted with equal quantities of distilled water to ensure that all samples contained the same protein content. SoyComil K samples were emulsified with 30% sunflower oil (v/v) using a Braun kitchen mixer (Kenwood LTD UK made) at speed 2. The sunflower oil was added at constant rate (1min) using a funnel, followed by emulsification with a Braun kitchen mixer at maximum speed for 30 sec. The emulsions were then homogenised using an APV homogeniser (APV Systems, Alberstlund, Denmark). A pressure of 500 bars was used for homogenisation and the process was carried out at room temperature (22 ± 3°C).

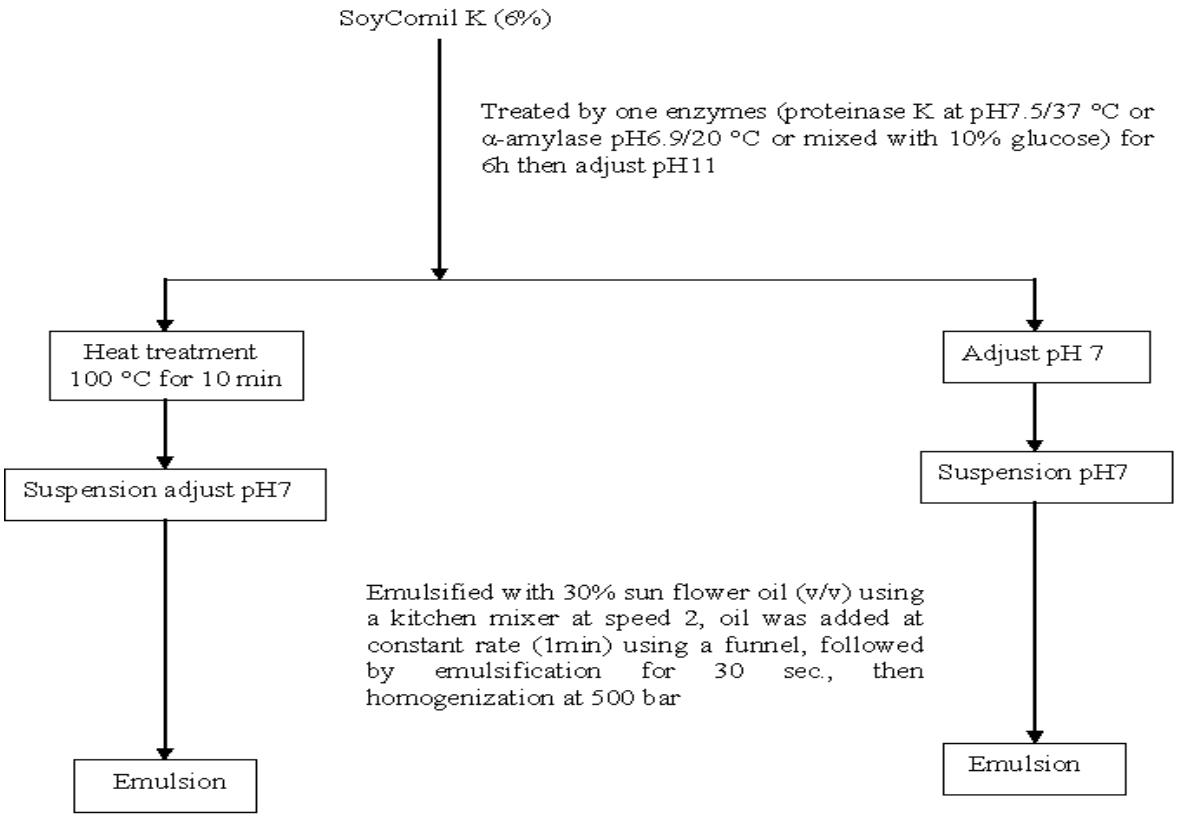


Figure 2.3 Procedure for preparation of SoyComil K emulsions

2.2.3.2 Soy protein isolates (SPI) emulsions (pH7)

Emulsions were prepared with heat-treated (95°C for 30 min) or non-heated SPI mixed with sugars as Figures 2.4. The protein concentration in emulsion was 4.3% and adjusted by adding the appropriate amount of water. The pH of the suspensions was adjusted to pH7 (measured by microprocessor pH meter, Hanna pH210, made in Romania). SPI samples were emulsified with 3% sunflower oil using a Braun kitchen mixer (Kenwood LTD UK made) at speed 2. The sunflower oil was added at constant rate, followed by emulsification at maximum speed for 30 sec. Half of the emulsions was not homogenized and the rest was homogenized using an APV homogenizer (APV System, Alberstlund, Denmark) at a pressure of 200-600 bars.

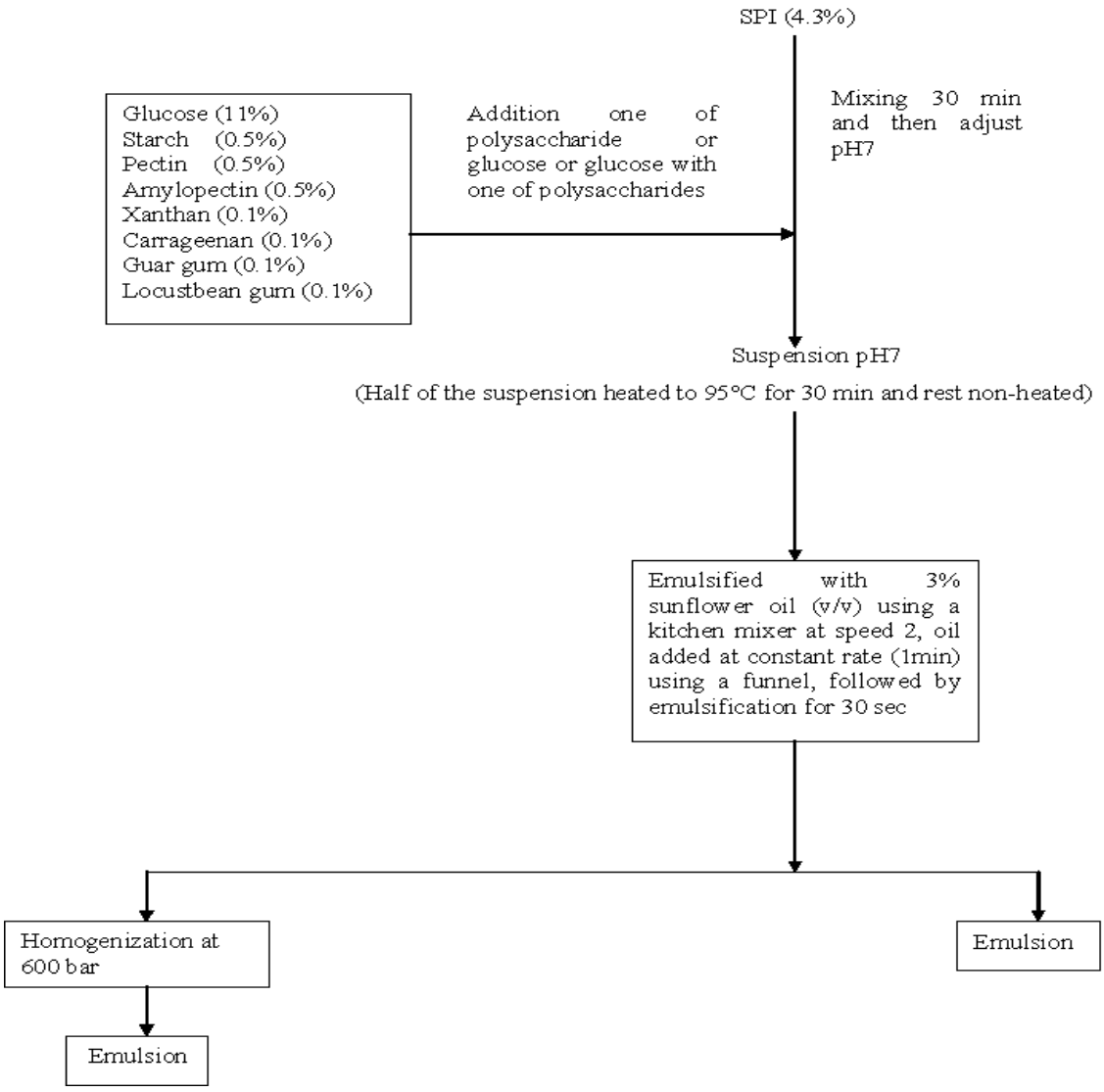


Figure 2.4 Procedure for preparation of SPI emulsions (pH7)

2.2.3.3 Soy protein isolates (SPI) emulsions (pH4.5)

Emulsions were made containing 20 % w/w sunflower oil, and 0.75-3% w/w of protein depending on the experiment. Protein was dissolved in imidazole buffer (0.05M). Mixed SPI/WPC emulsions were made at a total protein content of 3% w/w with ratios of SPI:WPC of 1:3, 1:1 and 3:1. SPI (3%) mixed also with different concentration (0.02, 0.04, 0.06, 0.08 and 0.1%) of polysaccharides (pectin, xanthan, carrageenan). The protein solution and oil were mixed initially using a Braun kitchen mixer (Kenwood LTD UK made) and then homogenized at 200 bar, room temperature (23 ± 3 °C) using an APV 2000 lab homogenizer. The emulsions were circulated through the homogenizer for 10 minutes prior to collection. This was to ensure as narrow a particle size distribution. The emulsions were then adjusted to a pH 4.5 (measured by microprocessor pH meter, Hanna pH210, made in Romania) using a 40% (w/v) glucono-δ-lactone (GDL) solution. The emulsions were left for 2 hours for the correct pH to be achieved. The correct volume of GDL required to achieve a set pH was determined by adding different volumes of GDL to protein solution and constructing a standard curve.

2.2.3.4 Whey protein emulsion (pH4.5)

Emulsions were prepared from whey protein concentrate (0.75%, 1.5%, 2.25% and 3% protein). Whey samples dispersed in 0.05M imidizole buffer and emulsified with 20% sunflower oil using a a Braun kitchen mixer (Kenwood LTD UK made) at speed 2. The sunflower oil was added steadily (1 min), followed by emulsification with a Braun kitchen mixer at maximum speed for 30sec followed by homogenization using an APV homogenizer (APV System, Alberstlund, Denmark). A pressure (200 bars) was used for homogenization, and the emulsion are re-circulated through the homogenizer for 10 min prior to collection, then adjust pH to 4 by GDL.

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2.2.4 Preparation of yogurts

Yogurts were prepared from emulsions (prepared as in section 2.2.3.2) by adjusting the pH to 4.5 (measured by microprocessor pH meter, Hanna pH210, made in Romania) using a 40% (w/v) glucono-δ-lactone (GDL) solution at room temperature (22 ± 3°C) and leaving them in the fridge at 5°C over night. The yogurts were prepared in plastic containers of 6mm diameter and 6 mm length as Figure 2.5.

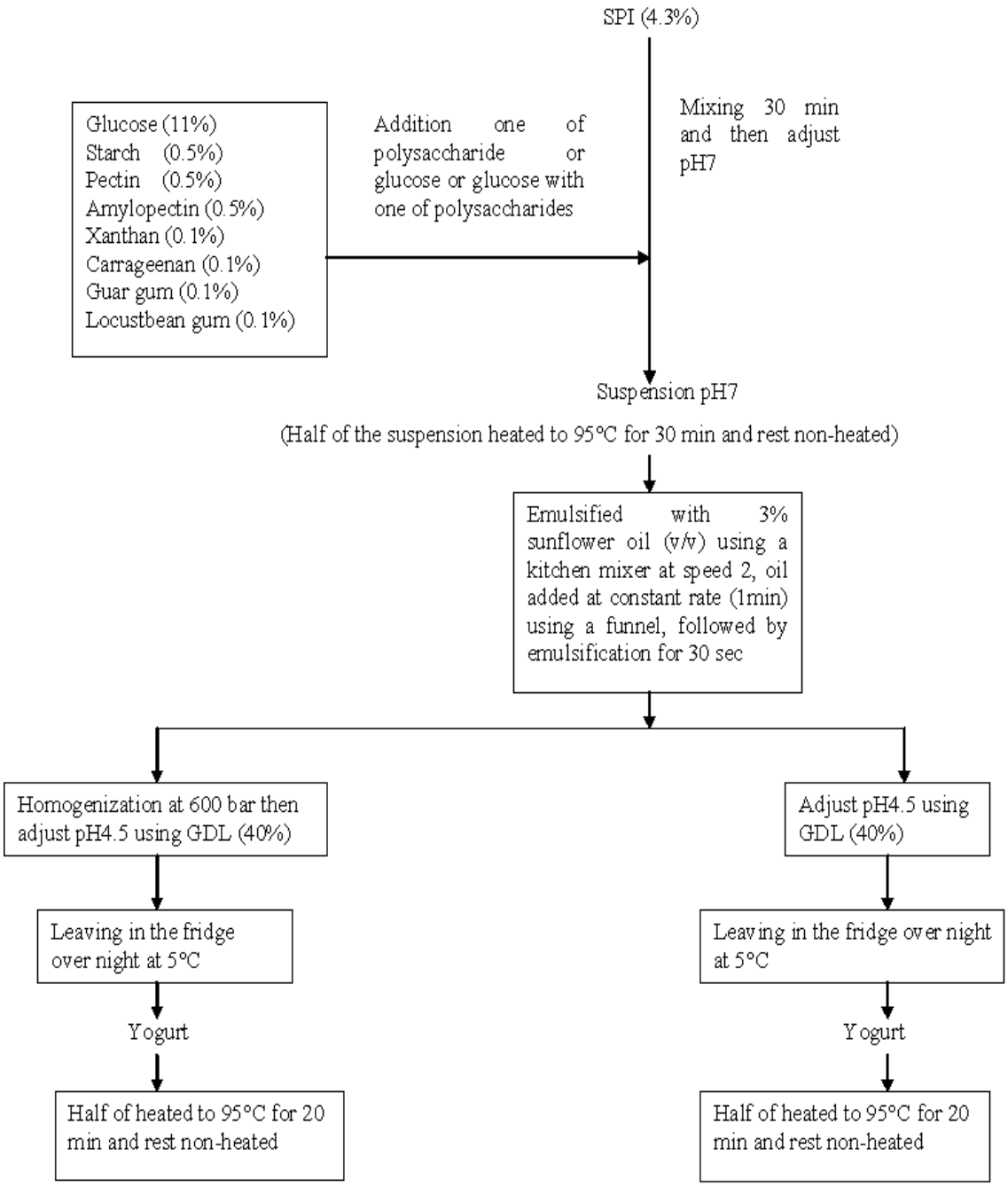

Figure 2.5 Procedure for preparation of yogurts

2.2.5 Determination of physico-chemical properties of soy protein

2.2.5.1 Differential scanning calorimetry (DSC)

The thermal characteristics of SoyComil K, commercial SPI, native laboratory prepared SPC and SPI were assessed by differential scanning calorimetry (DSC) using a DSC 2010 instrument (TA Instruments, New Castle, USA) according to the procedure of Sorgentini *et al*. (1995) and Renkema, (2001), with some modifications. Approximately 7mg of samples were placed in DSC hermetic aluminium pans. Analysis was performed at a temperature gradient of 20-140°C at a rate of 10°C/min. An empty aluminium pan was used as reference. The DSC data were analysed with universal analysis software (TA universal analysis, TA instruments).

2.2.5.2 Electrophoresis (SDS-PAGE)

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) of Soy Comil K and commercial SPI was performed according to the method of Laemmili (1970). Pre-cast native PAGE 10-20% Tris-glycine gradient gels were used in an electrophoresis unit (XCell Surelock™ Mini Cell, Invitrogen Life Technologies, Paisley, UK) at constant voltage 125V. Samples were diluted 1/15 (v/v) in distilled water, and were then dispersed in an equal volume of 2x SDS sample buffer (non-reducing sample buffer composed from 120mM Tris-HCl, pH6.8, 4% SDS, 20% Glycerol and 0.008% Bromophenol blue; while reduced sample buffer added 10% β-Mercaptoethanol). Running buffer was 10x SDS-PAGE buffer (1% SDS, 0.25M Tris-HCl and 1.92M Glycine per liter). The molecular weight markers (Plus2 pre-stained (1x) MW 4-250 KDa) and samples were run under reducing conditions (2-Mercaptoethanol), and non-reducing conditions.

2.2.5.3 Solubility

2.2.5.3.1 SoyComil K solubility

Soy samples were dissolved in milli-Q water at different protein concentrations (6, 10 and 18% w/v) with different pH (4.6, 6.5, and 9.0), and mixed by magnetic stirrer for 20 min. It was heated at different temperatures (room temperature, 50 and 80ºC) in a water bath for 10 min., and then cooled immediately on ice to 15°C. 10 ml of treated samples were centrifuged for 10 min at 3000 rpm using a centrifuge (Denley BS400, UK) at room temperature. The protein content of supernatant was determined in triplicate using the Bradford assay (Bradford, 1976). The total protein concentration was determined by the micro-Kjeldahl method (AOAC, 1980) using a Kjeldahl factor of 6.25.

$$\text{Solubility (\%)} = \frac{\text{Protein concentration in supernatant}}{\text{Total protein concentration in sample}} \text{ X } 100$$

2.2.5.3.1.1 Effect of salt (NaCl) on SoyComil K solubility

Soycomil (6%) at pH 9 (measured by microprocessor pH meter, Hanna pH210, made in Romania), was treated with different concentrations of NaCl (0.1, 0.2, 0.3, 0.4, and 0.5M) and heated to 80ºC for 10 min., followed by measurement of solubility.

2.2.5.3.1.2 Effect of some sugars on solubility of SoyComil K

Soycomil 6 %,(pH6.5), was mixed with different concentrations (1, 6, 7%) of sugars (sucrose, lactose, glucose) and heated to 70°C for 30 min. followed by measurement of solubility. SoyComil K was also mixed with 1% ribose (pH 9), and heated at different temperatures (80 and 100°C) for 2h, followed by measurement of solubility.

2.2.5.3.1.3 Effect of enzymes on solubility of SoyComil K

SoyComil K was treated with different carbohydrate hydrolysis enzymes (α-amylase at pH6.9 and temperature 20°C, hemicellulase at pH5.5 and temperature 37°C, cellulase at pH5 and temperature 37°C, pectinase K at pH4.5 and temperature 25°C and proteinase K at pH7.5 and temperature 37°C for different reaction times according to the manufacturer's instructions of Sigma campany in UK. Followed by heat treatment at different pH and temperatures, followed by measurement of solubility .

2.2.5.3.2 Solubility of soy protein isolate

Solubility of SPI (6%, pH7) was measured as described in section 2.2.5.3.1.

2.2.5.4 Turbidimetric measurement of soy protein aggregation

Turbidity of SoyComil K at different protein concentrations (6, 10, and 18% w/v), different pH (4.6, 6.5 and 9.0), and treated at different temperatures (RT, 50 and 80°C) for 10 min, was measured using a HACH 2100N Turbidity meter (CAMLAB). 200µl of sample was diluted 83-fold in aqueous solution (1% NaCl, 1% sucrose, 0.2% Tris). The samples were vortexed for about 5 sec. Blank measurements were recorded using buffer

solution only. Turbidity was measured using the single 90-degree detector, which receives the light scattered by the particles. Three replicate measurements were recorded and the results presented are the mean of these three replicates. Turbidity is expressed as NTU (nephelometric turbidity units).

2.2.5.5 Determination of hydrophobicity

The surface hydrophobicity of SoyComil K was determined using 1-anilinonaphthalene-8-sulphonate (ANS) assay according to the method of Kato and Nakai (1980), with some modifications (protein concentration and amount of hydrophobicity buffer). The relative fluorescence intensity of the ANS-protein conjugates was measured at room temperature (23± 3°C) using a fluorescence spectrometer Model 203, Norwalk, Connecticut, USA. The wavelengths of excitation and emission were 390 and 470 nm respectively. Quantification of ANS binding was carried out fluorimetrically by adding 15µl of ANS solution (8mM) to 1ml of diluted Soycomil K ranging in concentration between (0.3 - 0.5 mg/ ml). Hydrophobicity was expressed as the slope of the plot of fluorescence intensity values.

2.2.5.6 Determination of glycation degree

The degree of glycation of protein with different sugars was estimated by measuring the availability of primary amino groups, using ortho-phthaldiadehyde (OPA), based on the method of Chevalier *et al*, (2001) with some modifications (amount of buffers and protein concentration). The OPA reagent was prepared daily by mixing 80 mg of OPA (dissolved in 2ml of methanol), 100ml of 0.1M sodium borate buffer, pH9.3, 200mg of *N*-dimethyl-2-mercaptoethylammonium chloride (DMMAC), and 2.5 ml of 20% (w/v) SDS in 1 Litter of water.

2.2.5.6.1 Glycation degree of Soycomil K

100µl of heated (75ºC for 10 min) SoyComil K suspension (SoyComil K, 6% protein (pH 9.0) mixed with sugars “10% glucose, or 10% lactose, or 10% fructose, or 10% sucrose) was added to 2ml of OPA reagent. The absorbance was read at 340nm using a UV-VIS spectrophotometer (NovapecII, Amersham manufactured by Biochron Ltd. Cambridge England UK) after a minimal delay of 5 min. A calibration curve was obtained

by using 0.25-2mM Leucine as standard. SoyComil K 6% w/v (pH9) heated at 75ºC/ 10 min was used as control.

2.2.5.6.2 Glycation degree of soy protein isolate (SPI) in the presence of different sugars

SPI (4.3% protein, pH7) was mixed with 11% glucose, 0.5% starch, 0.5% pectin, 0.5% amylopectin, 0.1% xanthan, 0.1% carrageenan, 0.1% locust bean, and 0.1% guar gum respectively, and heated at 95°C for 30 min. Glycation was measured in the same manner as for of SoyComil K section 2.2.5.6.1.

2.2.6 Identification of intra-molecular bonds

Dispersing protein dispersions, emulsions or gels in solvents containing NaCl, 2-mercaptoethanol or urea can disrupt selective interactions among peptides. The method of Zhong *et al* (2006), Utsumi, and Kinsella (1985) was followed with some modifications (amount of protein and buffers). 3ml of sample was dispersed in 10 ml of the following buffers:

- 0.05 M Imidizole (as control).
- 0.3 M NaCl (electrostatic bonds breaking reagent).
- 0.2 M 2-mercaptoethanol (disulfide bonds bond breaking reagent).
- 8 M urea (hydrophobic bonds bond breaking reagent), and homogenized
- Using a Silverson (Model L2 R, made in England UK) for 1 min.

2.2.6.1 SoyComil K dispersions

3 ml of SoyComil K(6%, pH9) was dispersed in 10 ml of the buffers described above and one half of it was heated at (80°C for 10 min).

2.2.6.1.1 Particle size measurement

The particle size distribution ($D_{3,2}$, surface weighted mean; D3,2 = $\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of droplets of diameter d_i ; Frey, 2008) of solutions were measured using a Mastersizer 2000 (Malvern Instruments Ltd, Malvern, U.K.). D3,2 is defined as the diameter of a sphere that has the same volume/surface area ratio as a particle of interest or the ratio of the third to second moment of the probability density

function (Liu et al., 2007; Pacek et al., 1998; Downs and Sarv, 2003; chapter 2 section 2.2.8.1.1). The refractive index (RI) was set at 1.41, the x-ray absorbance was set at 0.001 and the laser obscuration was adjusted at about 5-10%. Reduction in particle size that should reflect the molecular forces contributing to maintenance of protein structure (Zhong *et al*., 2006). Triplicate measurements were carried out and the results presented are the mean of these three replicates.

2.2.6.2 SPI dispersions

Identification of bonds in 6% protein SPI dispersions, pH 9 was done as described above (section 2.2.6.1).

2.2.6.3 Soy yogurts

Yogurt samples (3g) were dispersed in 10 ml of buffers and homogenized using a Silverson (Model L2 R made in England UK) for 1 min. Solubility in the dispersion was then measured. The solubility of gel yogurt in the various solvents should reflect the molecular bonds contributing to maintenance of the gel network structure in yogurts (Utsumi and Kinsella, 1985). Triplicate measurements were carried out and the results presented are the mean of these three replicates.

2.2.7 Determination of free and total sulfhydryl (SH) groups

Sulfhydryl content was determined by using 5,5'-dithiobis(2-nitrobenzoic acid), according to Campbell *et al,*(2008).

Free SH:

For determination of free SH groups, 300µl of each sample was solubilised in 5ml of buffer containing 0.086 M Tris, 0.09 M glycine, 4 mM Na_2EDTA buffer, pH 8, followed by addition of 200µl of 20mM DTNB buffer. The solution was vortexed and allowed to stand at room temperature (23± 3°C) for 15 min. The absorbance was measured at 412 nm using a UV-VIS spectrophotometer (NovapecII, Amersham manufactured by Biochron Ltd. Cambridge England UK). The samples in buffer without DTNB were used as blanks

Total SH:

For determination of total SH group ,300µl of each sample was solubilised in 5ml of buffer containing 0.086 M Tris, 0.09 M glycine, 4 mM Na_2EDTA, 6 M urea and 0.5% SDS buffer pH 8; followed by addition of 200µl of 20mM Na_2DTNB buffer. The solution was vortexed and allowed to stand at room temperature for 15 min and absorbance was measured at 412 nm, the samples in buffer without DTNB were used as blanks.

2.2.7.1 SoyComil K dispersions

Sulfhydryl content of SoyComil K was determined that had been treated as in SoyComil K solubility (sections 2.5.3.1)

2.2.7.2 Yogurt

The amount of free and total sulfhydryl groups were analysed in yogurts prepared from:

- SPI
- SPI with glucose
- SPI and pectin
- SPI + pectin and glucose
- SPI and carrageenan
- SPI + carrageenan and glucose

2.2.8 Determination of emulsion properties

2.2.8.1 Emulsifying activity

The average oil droplet size distributions (D3,2 "surface weighted mean"; D3,2 = $\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of droplets of diameter d_i ; Frey, 2008) of emulsions were measured by laser light diffraction using a Mastersizer 2000 (Malvern Instruments Ltd, Malvern, U.K.). D3,2 is defined as the diameter of a sphere that has the same volume/surface area ratio as a particle of interest or the ratio of the third to second moment of the probability density function (Liu et al., 2007; Pacek et al., 1998; Downs and Sarv, 2003). The refractive index (RI) of particles was set at 1.41, the x-ray absorbance was set at 0.001 and the laser obscuration was adjusted at about 5-10%. Triplicate measurements were carried out and the results presented are the mean of these three replicates. Surface weighted mean D3,2 it represents the diameter whose ratio of volume to surface area is the same as that of the entire droplet sample (Liu et al., 2007; Downs and Sarv, 2003).

2.2.8.1.1 SoyComil K emulsions

The average droplet size distribution D(3,2) of SoyComil K emulsions, which had been prepared as described in section 2.2.3.1 was measured immediately after emulsion formation, after 10 days and 31 days .

2.2.8.1.2 SPI emulsions

SPI emulsions which had been prepared as described in section 2.2.3.2 with different sugars (11% glucose, 0.5% pectin, 0.5% amylopectin, 0.5% starch, 0.1% carrageenan, 0.1% xanthan, 0.1% guar and 0.1% locust bean). SPI (4.3%) was emulsified with 3% oil, followed by homogenisation at (200, 400, and 600 bar). Droplet size distribution (D3,2 and D4,3) of emulsions made was studied, before and after high-pressure homogenization, emulsions to under stand effect of homogenization on droplets size . In other hand changes in the average droplets size distribution (surface weighted mean "D3,2" and volume weighted mean "D4,3") of emulsions as function of SPI (heated or non-heated) to understand effect of heat treatment on droplet sizes, which reflect on water holding capacity.

2.2.8.2 Determination of destabilisation of emulsions

Emulsions were prepared from SPI with or without polysaccharides or whey, which had been treated as described in sections 2.2.3.3 and 2.2.3.4. The emulsions were pippetted into Eppendorf tubes and heated in water bath to different temperatures in different times, the tubes were removed at regular time intervals for different a period and quench cooled immediately by placing in ice as following:

- 60°C, tubes were removed every 5min for 60 min.
- 70°C, tubes were removed every 5min for 60 min.
- 80°C, tubes were removed every 1min for 10 min.
- 90°C, tubes were removed every 30sec for 5 min.
- 100°C, tubes were removed every 20sec for 3 min.

2.2.8.2.1 Calculation of emulsion destability

The kinetics of heat-induced breakdown of the emulsions was analysed using the methodology proposed by Euston et al. (Euston et al. 2000; 2001; 2002; Euston et al., 2001). In this method, the generalised rate equation (Eqn. 2.1) is assumed to describe the rate of change of the number of emulsion droplets (N) with time (t),

$$-\frac{dN}{dt} = k_n N^n \qquad (2.1)$$

Where k_n is the reaction rate constant and n the order of the reaction. This equation can be integrated to give a relationship between the number of emulsion droplets at the start of heating N_0 and at any time during the heating process, N_t, i.e.

$$\frac{N_t}{N_0} \left(\frac{(d_{3,0})_{t=0}}{(d_{3,0})_{t=t}} \right)^3 \qquad (2.4)$$

Where k is an apparent rate constant that depends on the initial number of droplets, i.e.

$$k = k_n N_0^{(n-1)} \qquad (2.3)$$

Before the apparent rate constant for heat-induced destabilization can be determined, the ratio N_t/N_0 and the order of the reaction n must be found. The ratio N_t/N_0 is related to the volume average diameter of the emulsion droplets (determined using the Mastersizer) by the equation,

$$\frac{N_t}{N_0} = \left[\frac{(d_{3,0})_{t=0}}{(d_{3,0})_{t=t}} \right]^3 \qquad (2.4)$$

For whey protein emulsions has found that the order of the heat-induced destabilization is n=1.5, suggesting a reaction that is a combination of than one unimolecular steps (Anema & McKenna, 1996). Diftis and Kiosseoglou, (2006b) have also found that the kinetics of soy-protein dextran emulsion heat stability is best described by a reaction of order n=1.5. Assuming n=1.5, a plot of $(N_t/N_0)^{-0.5}$ vs t should be linear with a slope equal to 0.5k. To correct the values of k determined from kinetic plots, we need to take account of the dependence on initial droplet number. We are unable to determine a value for N_0 directly from our particle size distributions. However, the relative initial droplet number ($N_0(T_1,c_1)/N_0(T_2,c_2)$) can be determined using a modified form of equation 4. For this we have chose to calculate this ratio relative to N_0 at 60°C and 0.75wt% protein. We are then

able to calculate a corrected value for k, i.e. $k_{1.5}$ using the relative initial droplet number in equation 3.

Analyse the temperature dependence of the reaction by using the Arrhenius equation. The reaction rate constant is related to the activation energy (E_a) for the reaction by the Arrhenius equation,

$$k_n = A.e^{-\frac{E_a}{RT}} \quad (2.5)$$

Where T is absolute temperature in degrees Kelvin, R the universal gas constant (8.3145 J/mol k), and A a pre-exponential factor. Plots of ln k_n vs 1/T are straight lines with a slope equal to $-E_a$. A thermodynamic analysis of the heat destabilization process can been carried out by calculating the free energy of activation (ΔG) using the Eyring equation,

$$k_n = \frac{k_b T}{h} e^{-\frac{\Delta G}{RT}} \quad (2.6)$$

and the enthalpy (ΔH) and entropy (ΔS) of activation using the thermodynamic relationships,

$$\Delta H = E_a - RT \quad (2.7)$$

$$\Delta G = \Delta H - T\Delta S \quad (2.8)$$

In equations (2.6)-(2.8) k_b is the Boltzmann's constant (1.381×10^{-23} $J.K^{-1}$) , h the Plank constant (6.626×10^{-34} J.S) and the temperature is in K.

2.2.9 Properties of yogurts

2.2.9.1 Water holding capacity of yogurts

Water holding capacity (WHC) was determined according to Unal *et al*., (2003) with some modifications. The samples were put into PE (Fisher Scientific TUL-750-036J) centrifuge tubes, and centrifuged for 20 minutes at 6400g at room temperature (23 ± 3°C). The supernatant was discarded, and pellet was weighed. WHC determined by using the following equation:

$$\text{Water holding capacity} = \frac{\text{Weight after centrifugation} - \text{Weight of empty sample tube}}{\text{Weight of sample}} \times 100$$

2.2.9.2 Texture analysis of yogurts

Texture parameters (hardness, cohesion strength, and adhesiveness) were determined by texture profile analysis using a texture analyzer Zwick/Roell (model BDO-FBO5. TS, Herefordshire, Germany) with a 20 mm diameter cylindrical probe. A force load of 0.1 N was used to penetrate the yogurt at a length of 40mm and crosshead speed of 10 mm forming.

2.2.10 Rheological measurements

Viscosity of SoyComil K emulsions prepared as described in section 2.2.3.1 was recorded on a Bohlin Gemini rheometer (Malvern Instruments Limited, Worcestershire, U.K.), using cone and plane geometry with a cone diameter of 40 mm and a cone angle of 4˚ (C4/40), after emulsion formation (maximum 20 min). The emulsion viscosity was monitored using controlled shear rate (0.0008 sec^{-1} – 55 sec^{-1}) and measurements were performed at room temperature. Viscosity was recorded at a frequency of 1 Hz with a fixed strain of 0.0008 and measured for 15 min. The sample was brought into the lower plate using a plastic spatula. The sample was forced to fill up the gap by lowering the upper plate down to the designated gap (150 mm) and the extra sample around the edge of the plate was trimmed with soft tissue. Measurements were carried out in triplicate and the mean values used were the average of the three replicates.

2.2.11 Confocal laser scanning microscopy

Microscopic images were obtained using a Leica DM-IRE2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with an Ar/He/Ne laser and 10 X objective lens (NPLAN 10 X 0.25 DRY). The fluorescence dye was excited at 50% of maximum adsorption at 543 nm and the detection bandwidth was set from 488 nm to 543 nm. Images were recorded at a resolution of 512 x 512 pixels and analysed by the manufacturer's software (Leica Software Development Kit, DM SDK, version 4.2.1), the labelling dye was Fluorescein (Nile blue A oxa zone, C2H18N2O2),

traces of the dye were added to the sample and dispersed throughout the samples. Samples were placed on a circular glass slide for analysis.

2.2.11.1 SoyComil K emulsions

Microscopic images of SoyComil K emulsions (of unheated and heated SoyComil K treated by enzymes "protease and α-amylase", or mixed with 10 % glucose) were studied.

2.2.11.2 Yogurts

. The microstructure of yogurt produced from SPI and pectin with glucose (highest hardness and WHC) and yogurt produced from SPI and carrageenan (lowest hardness and WHC) were studied.

2.2.12 Statistical analysis

All experiments were performed at least in triplicate using freshly prepared samples. Average and standard deviation were calculated from these triplicate measurements. Statistical differences between samples were calculated using student's t-test for independent samples (Microsoft Excel, Microsoft Corporation, designed in Redmond, Washington, USA).

Chapter Three

Functional properties of SoyComil K

3.1 Introduction

The rising cost of food-grade protein from animal sources, and growing food shortage in the world have increased the pressure on food scientists to develop new protein sources, particularly from vegetable proteins (Achouri et al., 2005). Soy protein ingredients must possess appropriate functional properties for food application and consumer acceptability. These are the intrinsic physicochemical characteristics which affect the behaviour of protein in food systems during processing, manufacturing, storage and preparation, e.g., solubility, gelation emulsification (Kinsella, 1979). Soy protein concentrate retains most of the fiber of the original soybean. Soy protein concentrate is widely used as functional or nutritional ingredient in a wide variety of food products, mainly in baked foods, breakfast cereals and in some meat products. (Kinsella, 1979). The solubility of protein is the most important factor determining its physico-chemical properties (Lakemond et al., 2000b). Protein solubilisation is the process of breaking interactions involved in protein aggregation, which includes disulfide bonds, hydrogen bonds, van der Waal forces, ionic interactions, and hydrophobic interactions. If these interactions are not prevented, proteins can aggregate or precipitate (Berkelman et al., 2004). Formation of insoluble precipitates of soy protein induced by heat occurs as a combination of two reactions: Reversible conversion of soluble monomers to aggregates followed by irreversible conversion into insoluble precipitates. The mechanism of protein thermo-coagulation is largely dependent on hydrophobic interactions among proteins (Nakai, 1983). Native soy protein has low solubility, because about 83% of the most non polar side chains (Ala, Val, Ile, Leu, Met, Phe, Trp, and Cys) and 82% of the peptide groups (-CO-NH-) are buried in the native state, consequently these are the most important groups that become exposed to solvent when protein is unfolded (denatured) (Pace et al., 2004). SoyComil K is a soy protein concentrate containing 72% protein, used as animal feed. Since this product is highly insoluble, its application is limited. This study has been undertaken to investigate ways to improve its solubility, as well as to understand the molecular basis of its insolubility.

3.2 Materials and Methods

Material and methods as described in chapter two

3.3 Results

3.3.1 Physicochemical properties of Soycomil K

3.3.1.1 SDS-PAGE electrophoresis

The SDS-PAGE technique was used to analyse the size distribution of the samples in terms of molecular weight (Romagnolo *et al*., 1990). Reducing and non-reducing SDS-PAGE of both supernatant and pellet of SoyComil K dispersions was done to analyse the soluble and insoluble fractions. Figure 3.1 illustrates the difference in electrophoretic migration of the soluble (supernatant) and insoluble (pellet) fractions under reducing and non-reducing conditions of SoyComil K, soy Arcon ® SJ (which is soluble kind of soy protein concentrate) and soy protein isolate (SPI) at pH7 and room temperature (Figures 3.2 and 3.3). In general, the intensity and numbers of bands decreased as solubility decreased in supernatants under non-reducing conditions (Figure 3.1 lanes 6, 7, 8, 9 and 10). There are 10 lanes in Figure 3.1, for lane 10 had eight detectable bands that were located at approximately 64, 50, 45, 36, 30, 16, 6, and 4 kDa. Some of these could be identified as the- subunit (64 KDa) and *β*-subunit (50-40 KDa) of *β*- conglycinin (Roesch *et al*, 2005, and Chove *et al.,* 2007). Acidic and basic polypeptides of glycinin corresponded 34-36 KDa, and 16-22 KDa bands respectively (Abtahi and Aminlari, 1997, and Roesch *et al*, 2005). Trypsin inhibitor of 2S fraction (whey protein) was located at 6-4KDa (Wolf, 1970), lane 9 is similar to lane 10, but with lower intensity and numbers of bands. There were other faint bands corresponding to 45, 35, and 22 KDa. These could be identified as *β*-subunit of *β*- conglycinin at 45 KDa. Lane 8 shows three bands that could be identified as follows: as α- and *β*- subunit of *β*- conglycinin at 64 and 50 KDa respectively, and basic polypeptides of glycinin at 22 KDa. There are many faint bands between 22-50KDa, which could be identified as acidic and basic polypeptides of glycinin. Lane 7 has lower intensity and numbers of bands than both lanes 9 and 10. Lane 6 this pellet has lower intensity and numbers of bands than the pellet of 1% SoyComil K (lane 8). Lane 5 is represented marker and lane 4 shows intense bands that could be identified as α- and *β*- subunit of *β*-conglycinin at 64 and 50 KDa respectively, and acidic and basic polypeptides of glycinin at 36, 16 KDa respectively. Lane 3 shows similar bands than lane 4 (supernatant of 1% SoyComil K) but with lower intensity. Lane 2 was similar to lane 4 in number of bands but

with less intensity. Lane 1 shows one band that could be identified as the basic polypeptide of glycinin at 22 KDa, and the two faint bands could be identified as α- subunit and *β*- subunit of *β*- conglycinin at 64 and 50 KDa respectively.

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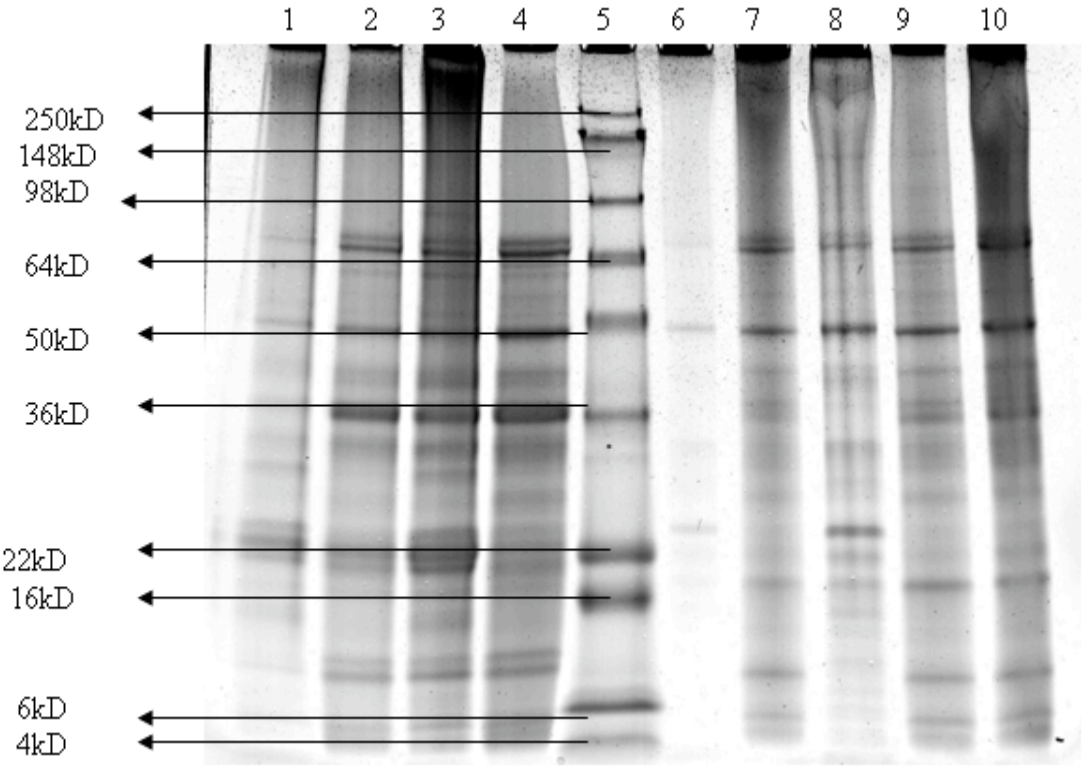

Figure 3.1 Reducing and non-reducing SDS- PAGE of supernatant and pellet SoyComil K pH 9.0 treated at different temperatures.

Where Lane 1: The pellet of 6% SoyComil K, (pH9) that was heated to 100°C for10 min under reducing condition.

Lane 2: The 30% soluble supernatant (6% SoyComil K, pH9) that was heated to 100 °C for10 min under reducing condition.

Lane 3: The pellet of 1% SoyComil K (pH9), heated to 80 °C for10 min under reducing condition.

Lane 4: The 64.5% soluble supernatant of 1% SoyComil K (pH9) heated to 80 °C for 10 min under reducing condition.

Lane 5: Marker

Lane 6: The pellet of 6% Soy Comil K (pH9) that was treated to100 C° for10min under non-reducing condition.
Lane 7: The 30% soluble supernatant of 6% SoyComil K, (pH9) that was heated to 100 °C for10min under non-reducing condition.
Lane 8: The pellet of 1% SoyComil K (pH9) that was heated to 80°C for10min under non-reducing condition.
Lane 9: The 64.5% soluble supernatant of 1% SoyComil K (pH 9) heated at 80°C for10min under non-reducing condition.
Lane 10: The 100% soluble supernatant of 1% SoyComil K (pH9) that was heated to 100°C for10min under non-reducing condition.

Non-reducing SDS- PAGE of soy protein concentrates (SoyComil K and soy Arcon ® SJ) and Soy protein isolates pH 7 at room temperature (Figure 3.2) represented 4 lanes. Lane 4 is marker; lane 3 had one intense band that is located approximately 50 KDa, and three faint bands that were located at approximately at 64, 22 and 16 KDa. Some of these could be identified as as α- and *β*- subunit of *β*- conglycinin at 64 and 50 KDa respectively. Basic polypeptidees of glycinin correspond 16-22 KDa. Lane 2 had six detectable bands that were located at approximately 64, 50, 36, 16, 6, and 4 kDa. Some of these could be identified as the α- subunit (64 KDa) and *β*-subunit (50 KDa) of *β*- conglycinin. Acidic and basic polypeptides of glycinin corresponded 36 KDa, and 16 KDa bands respectively. Trypsin inhibitor of 2S fraction (whey protein) was located at 6-4KDa. Lane 1 is similar to lane2 of Figure 3.2, but more intensity.
Reducing SDS- PAGE of soy protein concentrates (SoyComil K and soy Arcon ® SJ) and SPI pH 7 at room temperature (Figure 3.3) show 4 lanes. Lane 4 is marker; lane 3 shows very intense defused integrated bands that could be identified as α- and *β*- subunit of *β*- conglycinin at 64 and 50 KDa respectively, and acidic and basic polypeptides of glycinin at 36, 22 KDa respectively. There were two bands at 6 and 4KDa that could be identified as trypsin inhibitor of 2S fraction (Wolf, 1970). Lane 1 was similar to lane 3 of Figure 3.3, but with higher intensity. Lane 1 was similar to lanes 2 and 3 of Figure 3.3, but more intensity.

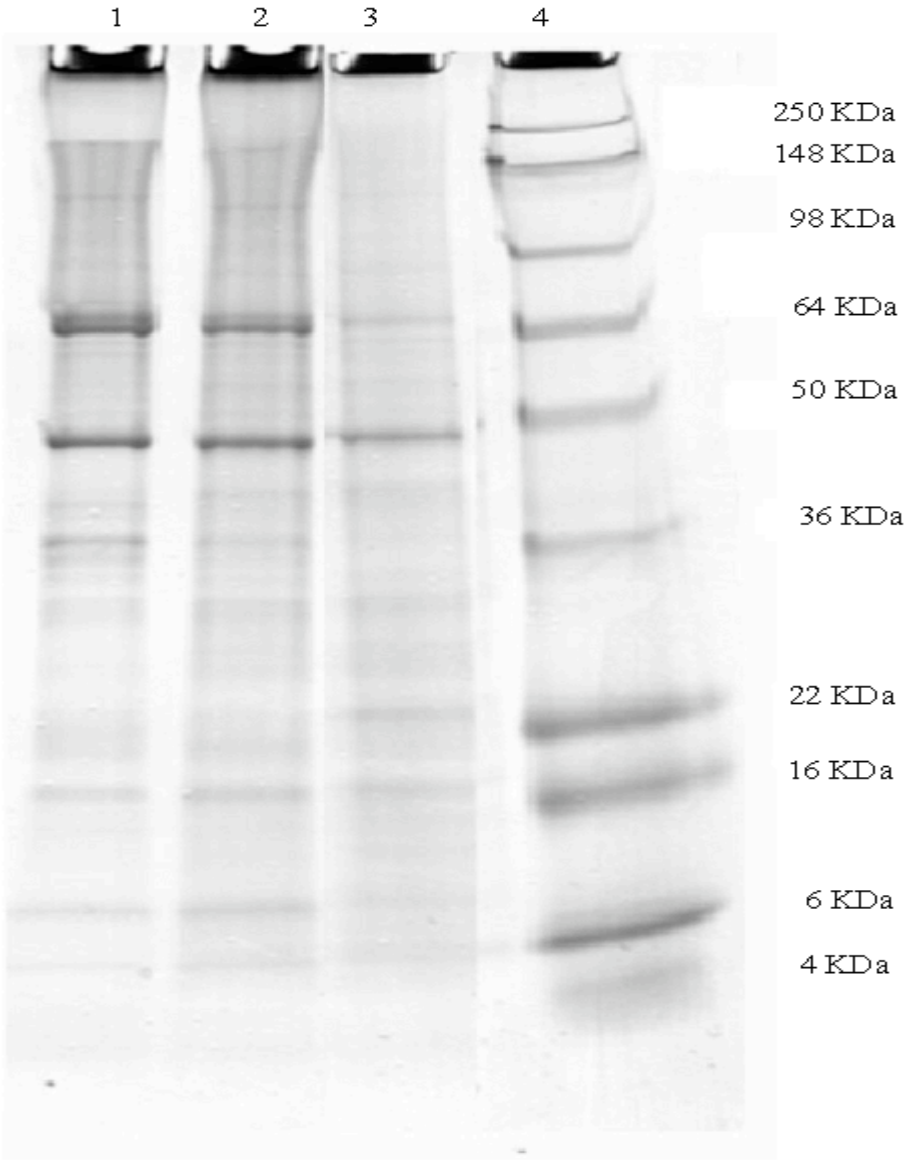

Figure 3.2 Non-reducing SDS- PAGE of soy protein concentrates (SoyComil K and soy Arcon ® SJ) and Soy protein isolates pH 7 at room temperature.

Where: Lane 1: Soy protein isolates (SPI) 6% (pH7) and solubility 13.8% solubility.

Lane 2: Soy protein concentrates 6% (soy Arcon ® SJ), pH7 with solubility of 7.6%.

Lane 3: SoyComil K 6% (pH7, room temperature) with solubility of 0.9%.

Lane 4: Marker.

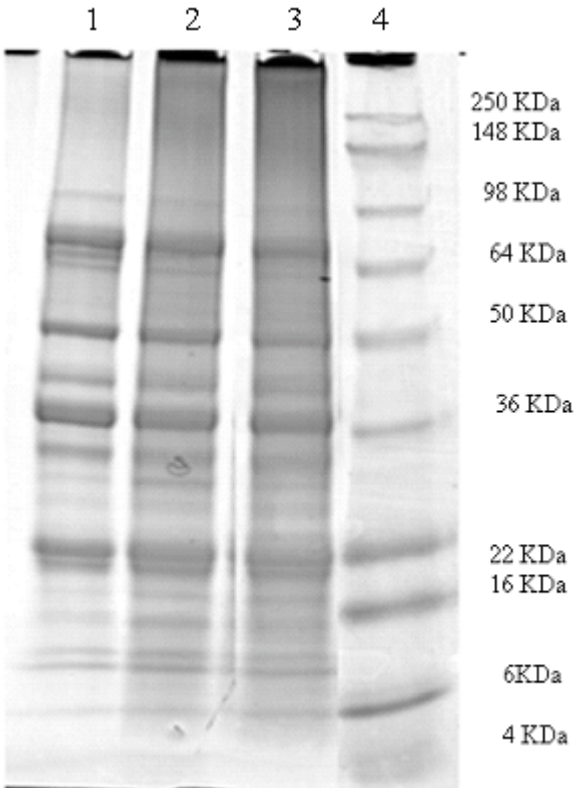

Figure 3.3 Reducing SDS- PAGE of soy protein concentrates (SoyComil K and soy Arcon ® SJ) and SPI pH 7 at room temperature

Where: Lane 1: Soy protein isolates (SPI) 6% (pH7) and solubility 13.8% solubility.

Lane 2: Soy protein concentrates 6% (soy Arcon ® SJ), pH7 with solubility of 7.6%.

Lane 3: SoyComil K 6% (pH7, room temperature) with solubility of 0.9%.

Lane 4: Marker.

3.3.1.2 Differential scanning calorimetry (DSC)

The thermal characteristics of SoyComil K and native laboratory prepared SPC were determined by DSC. The DSC thermogram of native SPC (Figure 3.4) shows two exothermic transitions peaks at 75.24°C with Δ*H* 113.6 J/g and 94.55°C with Δ*H* 2.987 J/g, which correspond to the thermal denaturation of *β*- conglycinin and glycinin respectively (Hua *et al*., 2005). SoyComil K also exhibited two exothermic transition peaks, but at 74.72°C with Δ*H* 98.5 J/g and 102.79°C with Δ*H* 0.8352 J/g (Figure 3.5), which correspond to the thermal denaturation of *β*- conglycinin and glycinin respectively. Glycinin of SoyComil K had a higher denaturation temperature than laboratory prepared SPC.

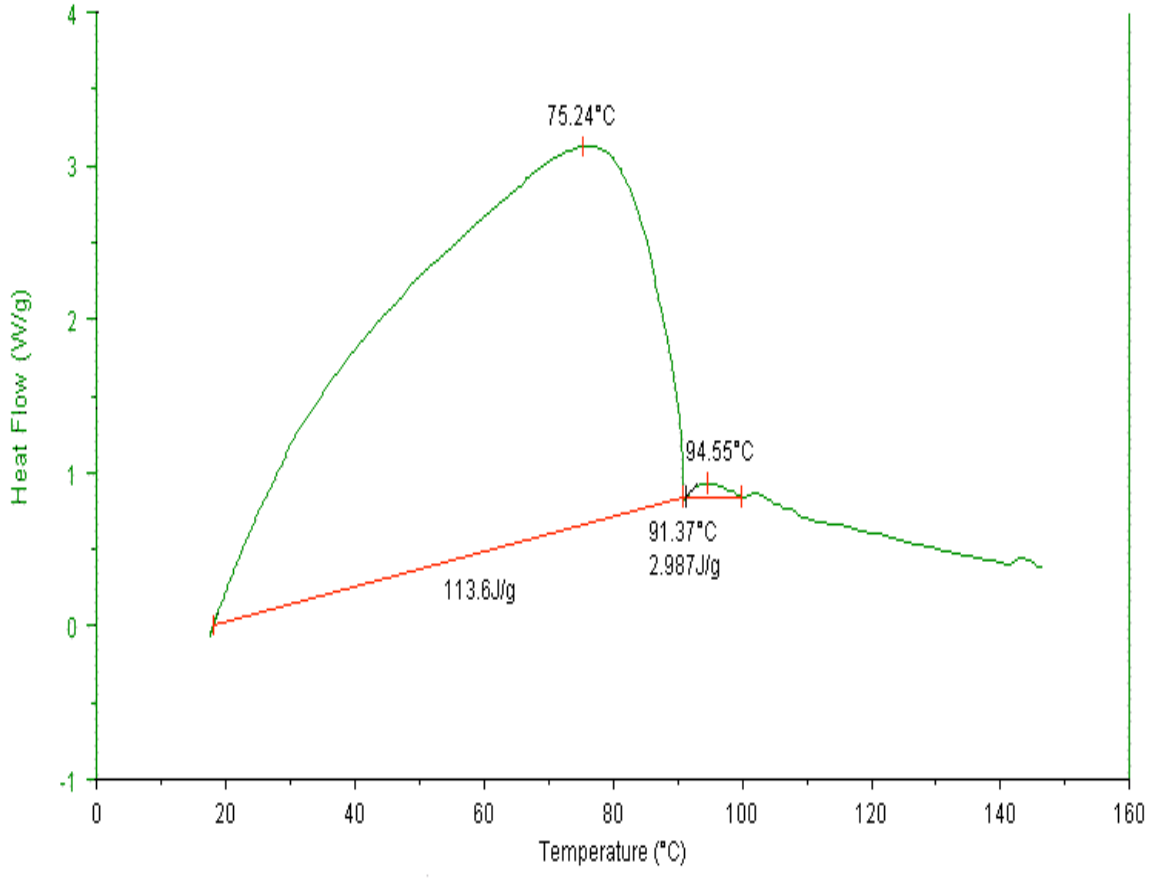

Figure 3.4 DSC thermogram of native laboratory prepared SPC.

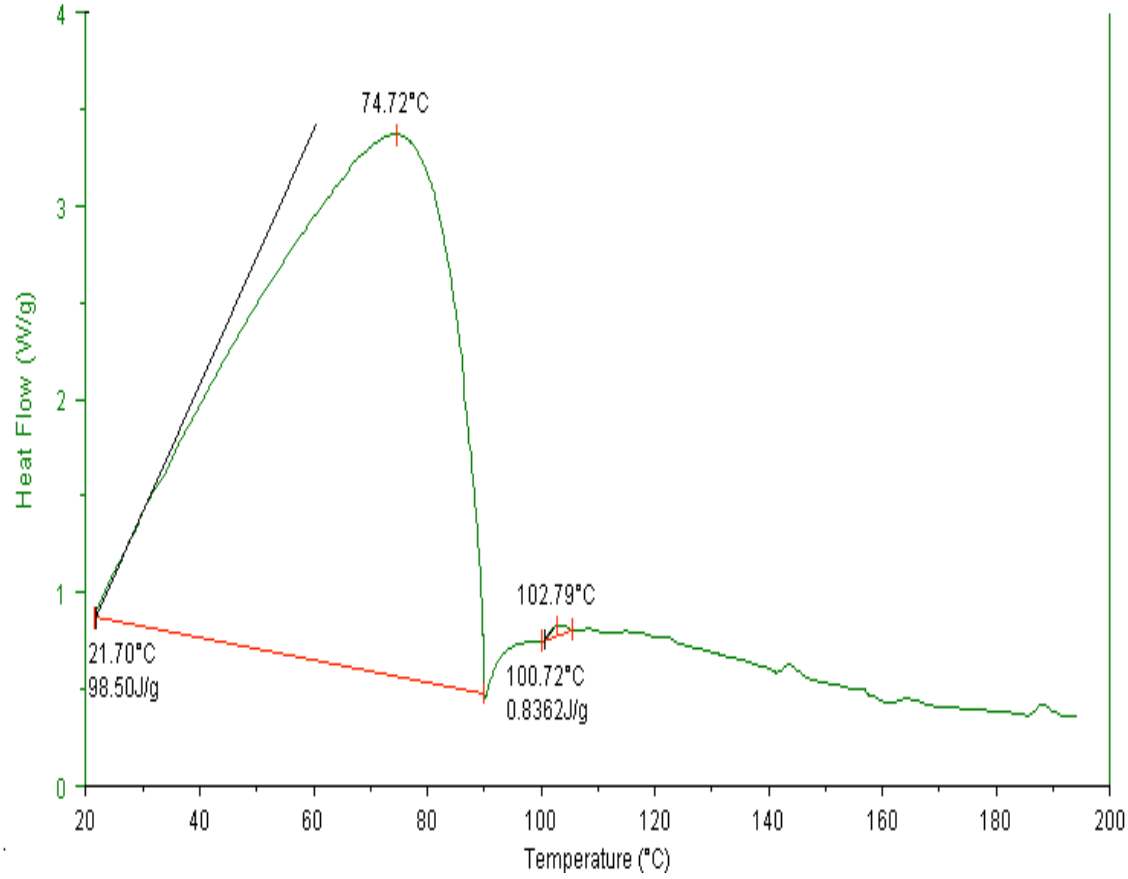

Figure 3.5 DSC thermogram of SoyComil K

3.3.2 Factors influencing Soycomil K solubility

3.3.2.1 Effect of pH, temperature and protein concentrations on SoyComil K solubility

The results of heating (room temperature "22± 3°C", 50 °C and 80 °C) SoyComil K at different concentrations (6, 10 and 18% w/v) and pH (pH4.6, pH6.5 "control" and pH9.0) at different temperatures for 10 min (Figure 3.6). Show that the highest increase in solubility was obtained at concentration 6%, 80°C and pH 9.0 was 8.24%, where the lowest increase in solubility was at pH 4.6, concentration 18% and room temperature was 0.11%. Heating at 80°C resulted at the highest increase in solubility for all samples compared to solubility of all samples at room temperature. Under all experimental conditions the highest solubility was obtained with 6% protein, whereas 18% protein showed the lowest solubility

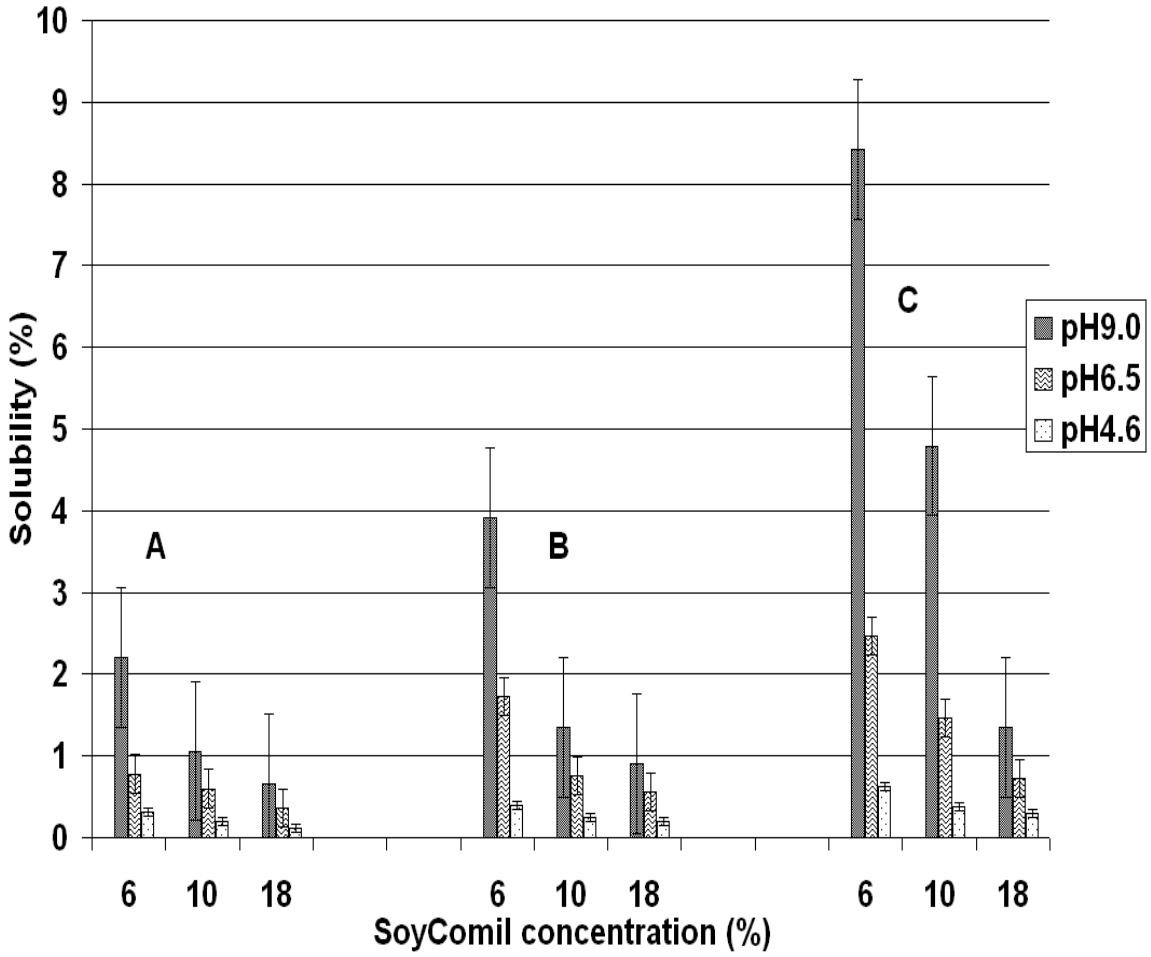

Figure 3.6 Effect of pH, protein concentrations and heat treatment on SoyComil K solubility

Where A: treatment at room temperature (22± 3°C), B: treatment at 50 °C and C: treatment at 80 °C.

3.3.2.2 Effects of pH, temperature and concentration on turbidity of SoyComil K

The turbidity intensity was used as a measure of aggregation of proteins; the higher the turbidity, the more protein aggregation occurred (Ker and Chen, 1998). In this study the effect of heating SoyComil K at different concentrations (6, 10 and 18% w/v), pH (4.6, 6.5 "control" and 9) and temperatures (room temperature, 50 and 80°C) for 10 minutes on turbidity was measured. Results in Figure 3.7 show the highest increase in turbidity was at pH 4.6, concentration (18%) and room temperature was 240.1 NTU, whereas the lowest increase in turbidity was at concentration (6%), 80°C and pH 9.0 was

54.88 NTU. Under all experimental conditions, the highest turbidity was obtained with 18% protein, whereas 6% protein showed the lowest turbidity. Non-heated SoyComil K had higher turbidity than heat-treated (Figure. 3.7). Thermal treatment of SoyComil K decreased turbidity.

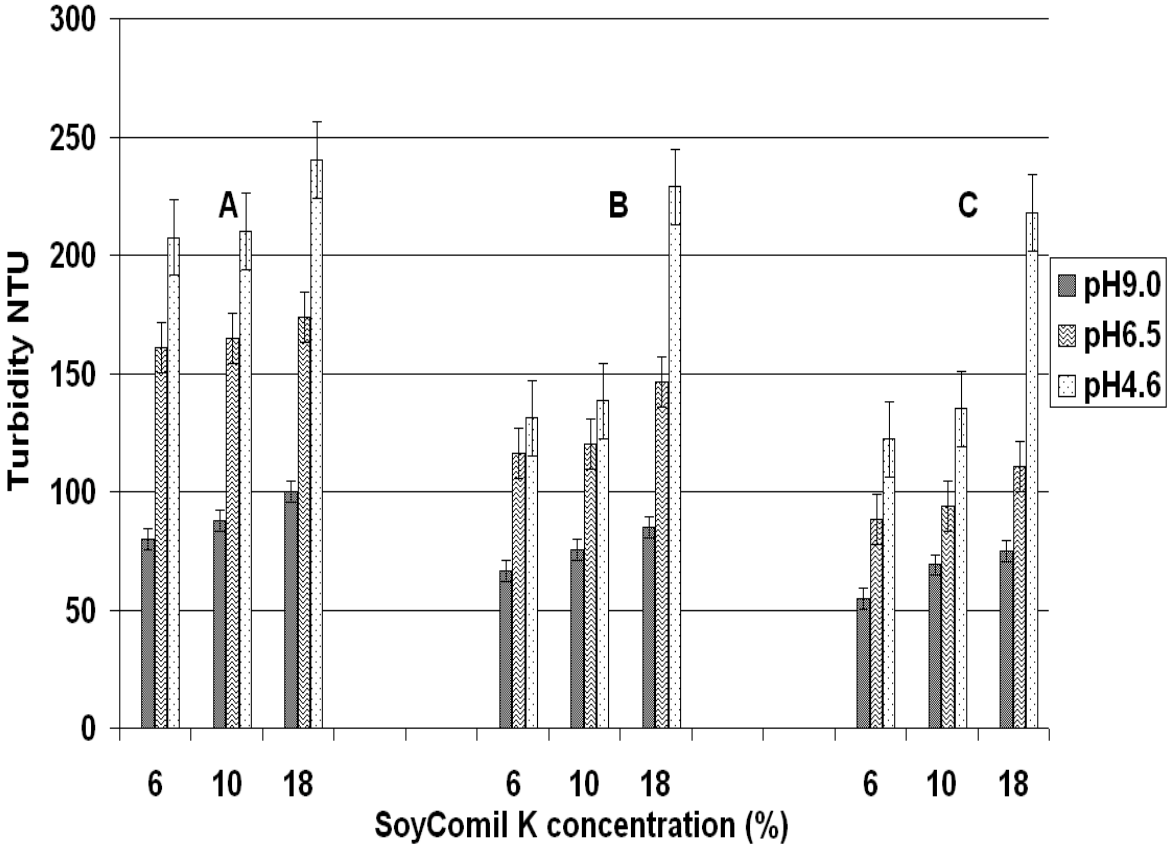

Figure 3.7 Effect of pH, protein concentration and heat treatment on SoyComil K turbidity

Where A: treatment at room temperature (22± 3°C), B: treatment at 50 °C and C: treatment at 80 °C.

3.3.2.3 Effect of salt (NaCl) on SoyComil K solubility

Figure 3.8 shows the solubility of 6% (protein) SoyComil K pH 9.0 containing different NaCl concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 M) after heat treatment at 80°C for 10 min. compared to the control containing only Soycomil K. The graph shows that:

The highest increase in solubility (9.6%) was with 0.1M NaCl due to salting-in, whereas the solubility decreased as NaCl increased. At 0.5 M NaCl the solubility decreased (8.19%) less than control which has solubility 8.42% due to salting-out.

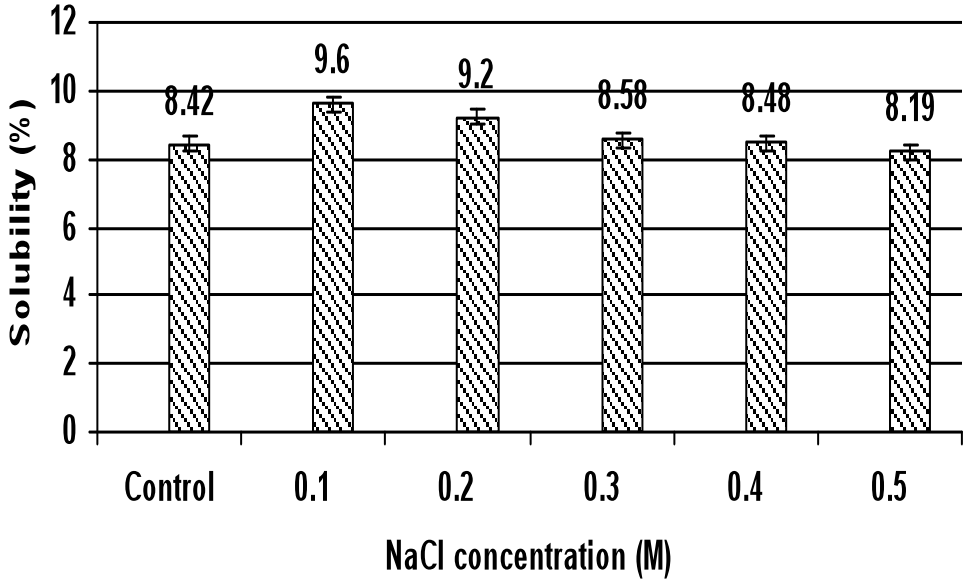

Figure 3.8 Effect of salt (NaCl) on solubility of SoyComil K pH9 at 80°C for10 min

3.3.2.4 Effect of some sugars on solubility of SoyComil K

Solubility of SoyComil K (6% protein pH 6.5) treated with different sugars (sucrose, glucose, and lactose), at different concentrations (1, 6, and 7%) at different temperatures (70°C for 30 min and 100°C for 10min) is presented in Figures 3.9 and 3.10. Heat treatment (70°C for 30 min) of SoyComil K with sugars caused increase solubility. Figure 3.9 shows that there was a significant difference (confidence level of 95% $p<0.05$) between sugars' effect on solubility of SoyComil K. the highest solubility was obtained with sucrose in all concentrations. The difference in solubilities caused by sucrose and lactose was not significant, whereas solubility caused by glucose was significantly lower than that caused by sucrose and lactose. Heat treatment (100°C for 10min) of SoyComil K with sugars cause decrease solubility due to Maillard reaction. Fig 3.10 shows the effect of heat treatment of (6% SoyComil K (pH 6.5) in the presence of 1% (sucrose, lactose, and

glucose) at 100°C for 10 min on solubility. Overall the solubility of SPI by heat treatment at 100°C for10 min decrease solubility due to maillard reaction, while at 70°C increase solubility due formation of hydrophilic layer around the protein.

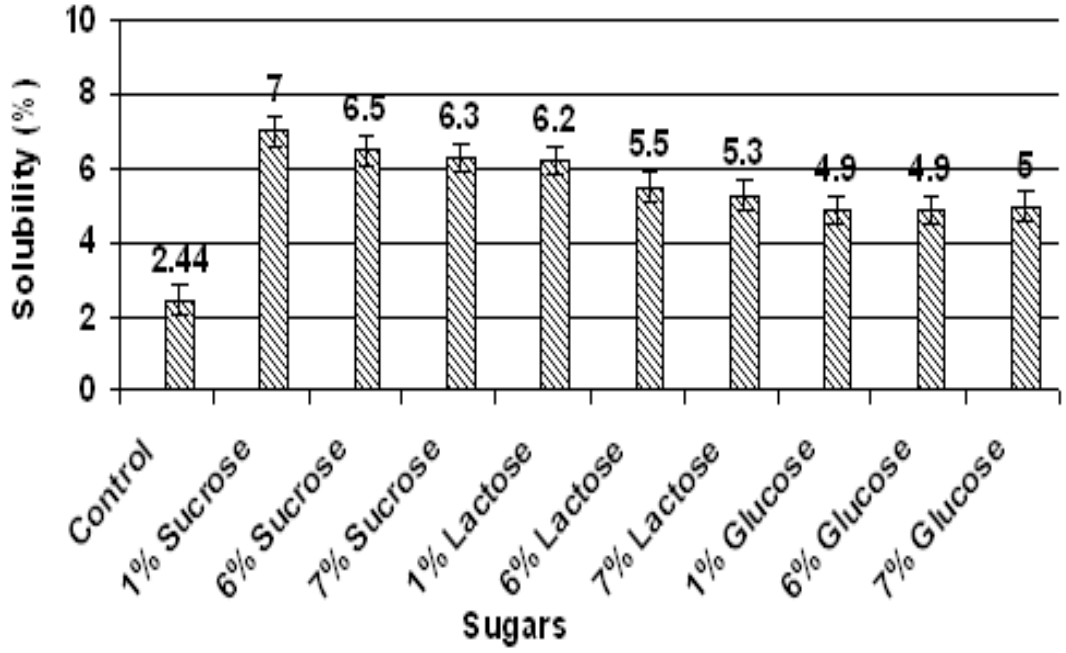

Figure 3.9 Effect of different sugars on solubility of 6% SoyComil K pH6.5 at 70°C for 30min

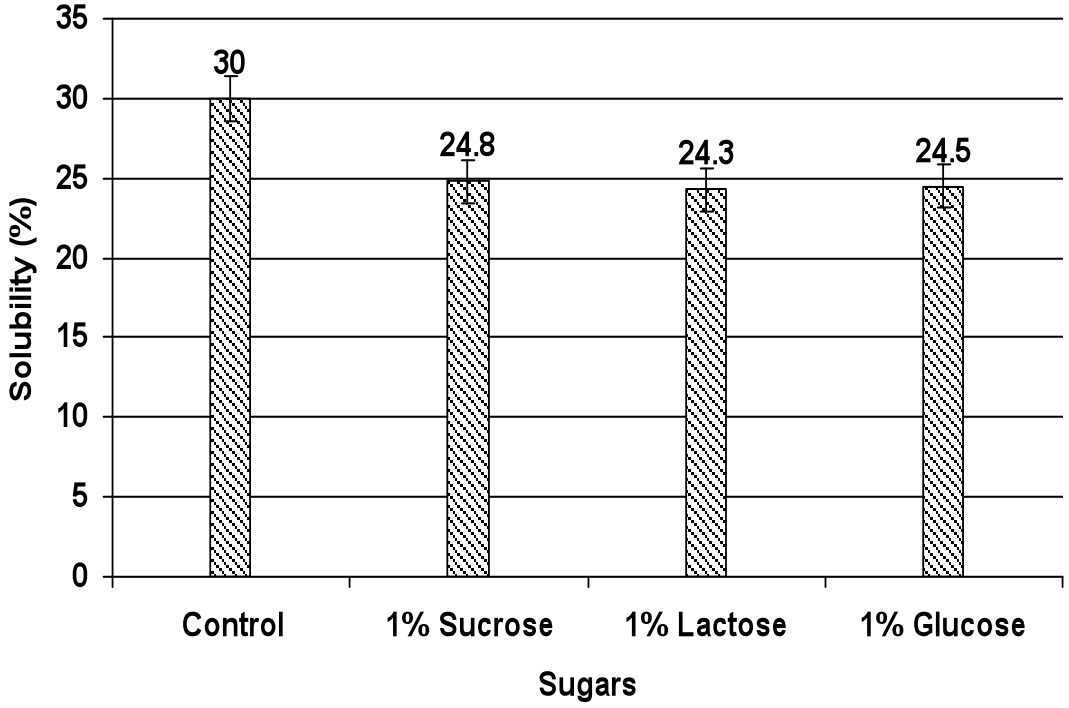

Figure 3.10 Effect of different sugars on solubility of 6% SoyComil K pH6.5 at 100°C for 10min

3.3.2.4.1 Glycation degree of SoyComil K

Suspensions of SoyComil K (6%), pH 9 containing different types of sugars (10% glucose, 10% fructose, 10% lactose and 10% sucrose) were heated 70°C for 30 min. The control was SoyComil K (6%) without sugar. The effect of heating in the presence of different sugars on the free amino groups concentration is presented in Figure 3.11. The free amino acid content of SoyComil K was expressed in terms of L-leucine. The result shows that there was no significant difference in glycation degree between different sugars. Figure 3.11 shows decrease in free amino groups caused by all sugars and highest decrease was obtained by glucose (0.532 µg/ µl), which mean highest glycation, where lowest glycation obtained with sucrose (0.5886 µg/ µl).

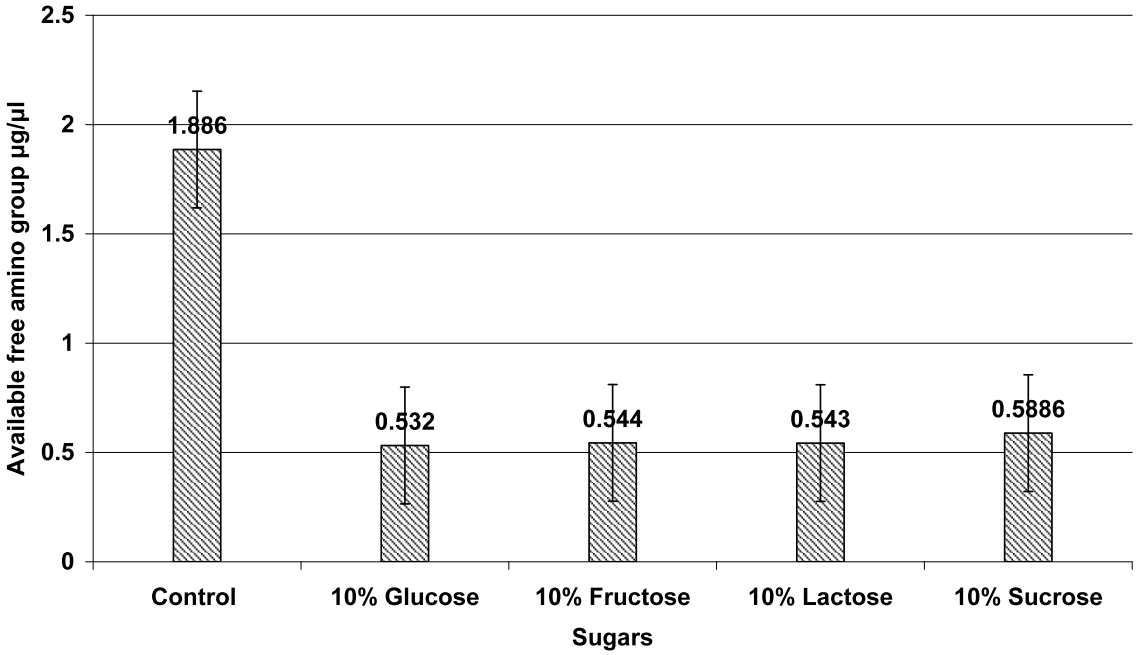

Figure 3.11 Availability of free amino groups of SoyComil K 6% pH9 treated at 75ºC for 10min with different sugars

3.3.2.5 Effect of enzymes on solubility of SoyComil K

SoyComil K (6% w/v) was treated with various carbohydrate hydrolysis enzymes (α-amylase, hemicellulase, cellulase and pectinase) and also with a proteinase K. Proteinase K is an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. The Proteinase K is classified as a serine protease. The smallest peptide to be hydrolyzed by this enzyme is a tetrapeptide (Ollar, 1999). Highest solubility after treated by carbohydrate hydrolysis enzymes was obtained with α-amylas. Treatment with α-amylase significantly increased solubility of Soycomil K, the solubility increased from 8.42% at pH 9, 80 °C for 10 min to 12.82% (Figure 3.12) and from 12.5% at pH9, 80 °C for 1h to 15% (Figures 3.13). Increase in solubility by hydrolysis by cellulose and hemicellulase was not significant (Figure 3.12), where hydrolysis by pectinase decreased solubility to 1.29% (Figure 3.12).

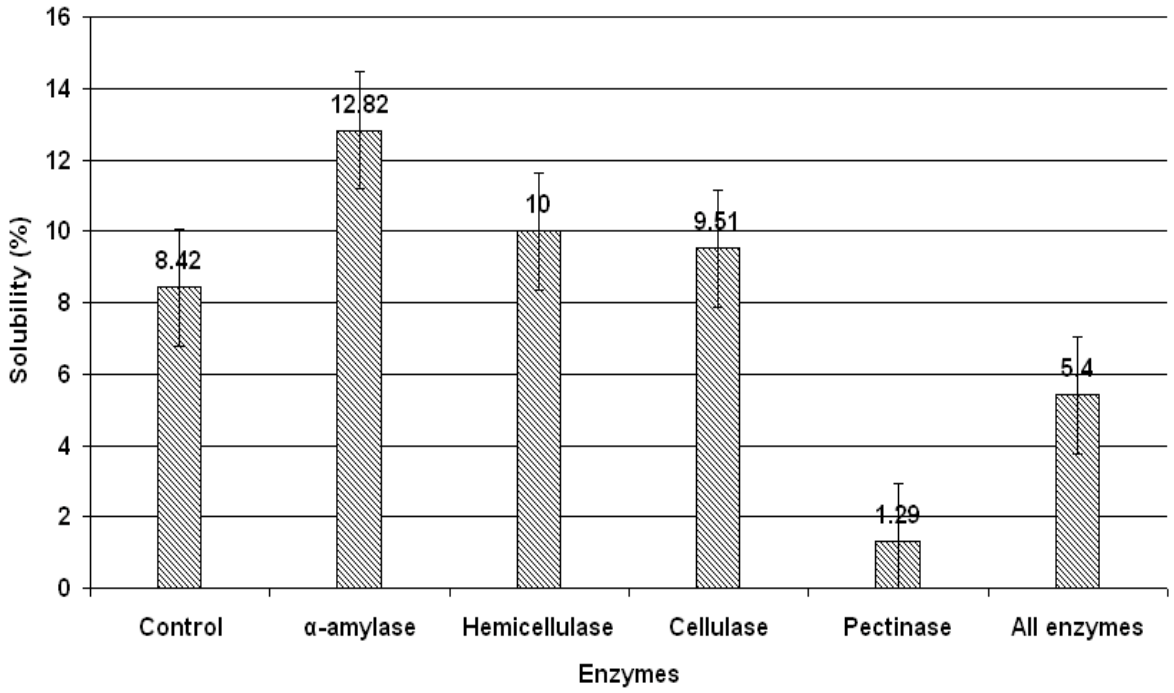

Figure 3.12 Effect of enzymes on solubility of 6% SoyComil K . SoyComil K was mixed with enzymes at optimum pH, temperature and time for each one, and then adjusted to pH 9 and heated to 80°C for 10 min

To further increase solubility, both heating and mixing time of SoyComil K (pH9), 80°C with α-amylase was increased. Figure 3.13 shows solubility increased with incubation time (6 hours) from 12.5% to 15% and increasing mixing time to 24 hours cause increased solubility from 12.5% to 17.1%. however, increasing the temperature to 100°C, after hydrolysis by α-amylase, showed no effect on solubility with 6hour mixing time, yet mixing SoyComil K with α-amylase for 24 hours caused solubility to decrease from 30% to 24% (Figure 3.13).

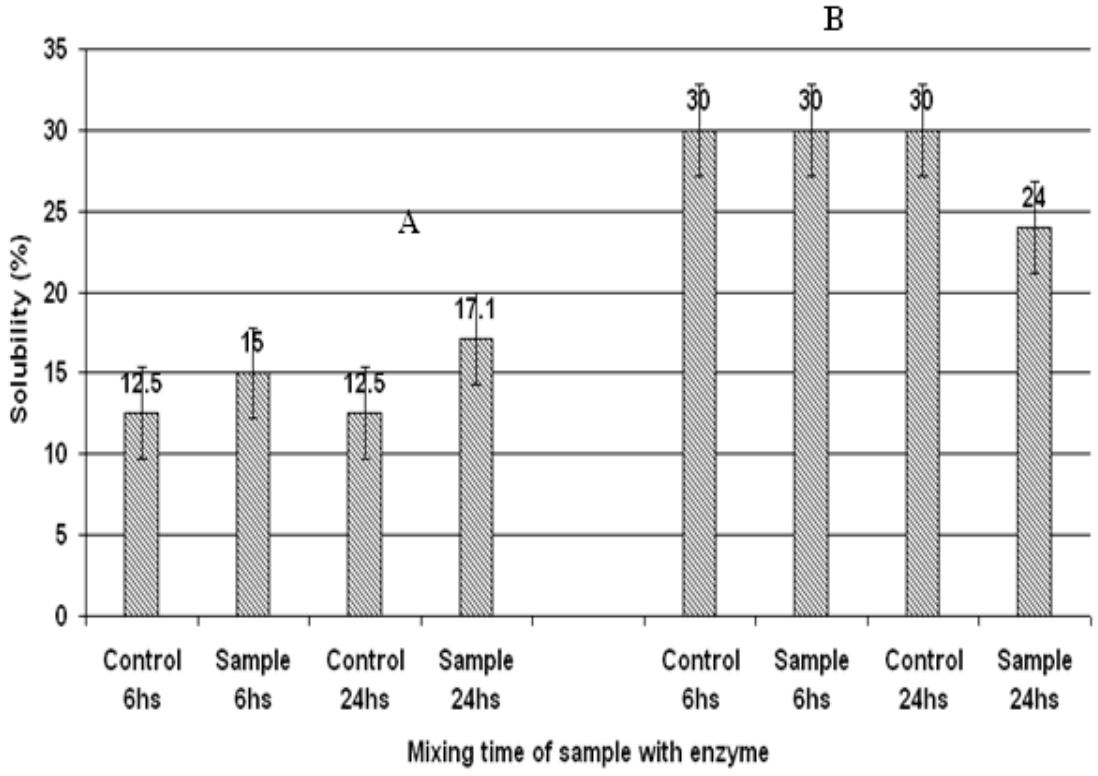

Figure 3.13 Effect of mixing time with α-amylase on solubility of 6% SoyComil K. SoyComil K was mixed with α-amylase at optimum pH and temperature then adjust pH to 9.0 and heated at different temperature for 1h

Where A: heat treatment at 80°C and B: heat treatment at 100°C

To increase solubility SoyComil K treated by proteinase K followed by heat treatment. Results show there is increased in solubility of SoyComil K from 15.5% to 20.8% at pH9 and 80°C , but at high temperature (100°C) solubility decreased from 30% to 20.1% (Figure 3.14).

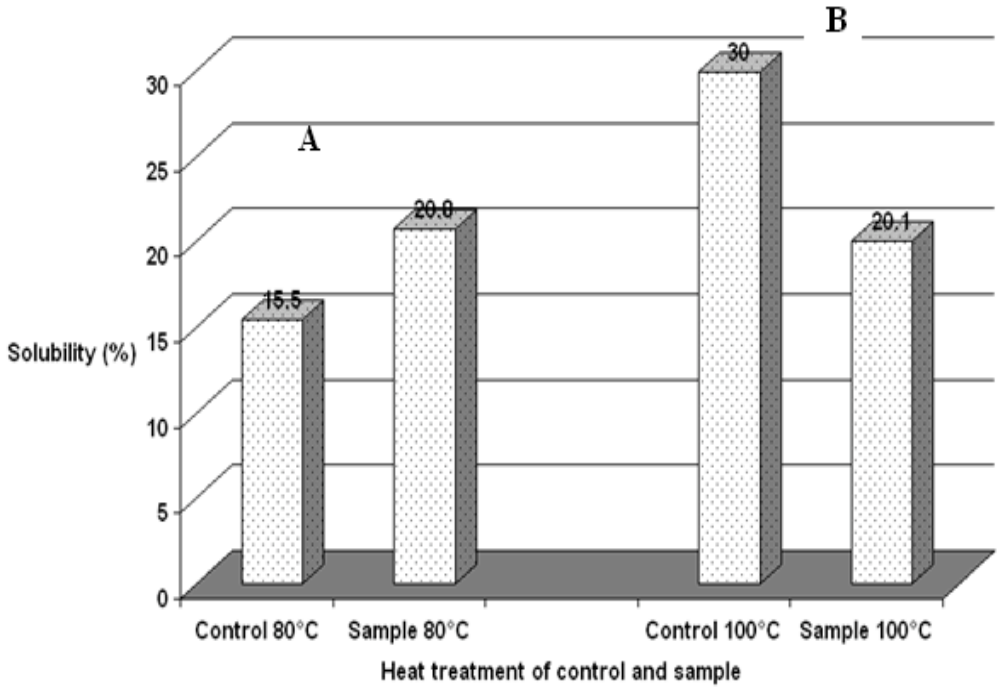

Figure 3.14 Effect of proteinase K on solubility of 6% SoyComil K. SoyComil K was mixed with proteinase K at optimum pH (7.5) and temperature (37°C) then adjust pH to 9 and heated at different temperature for 1h

3.3.2.6 Intramolecular bonds in Soycomil K dispersions

3.3.2.6.1 Effect of solubilisation in different buffers on particle size of Soycomil K

Dispersing protein in solvents containing (0.3M NaCl, 0.2M 2-mercaptoethanol, and 8M urea) can disrupt selective bond among peptides, which could lead to reductions in particle size or decreases in molecular weight averages (Zhong *et al.*, 2006). A decrease in average particle size was found with the addition of each of the three reagents (0.3M NaCl, 0.2M 2-mercaptoethanol, and 8M urea) in both unheated and heated samples (Figure 3.15). In all samples, the smallest particle size was obtained with sample treated by 8 M urea, which indicates that the majority of bonds were hydrophobic bonds. Larger particle sizes were obtained with disulfide bond reducing reagent (2-mercaptoethanol) and the largest

particle size was obtained with the sample treated by 0.3 M NaCl, which disrupts electrostatic bonds.

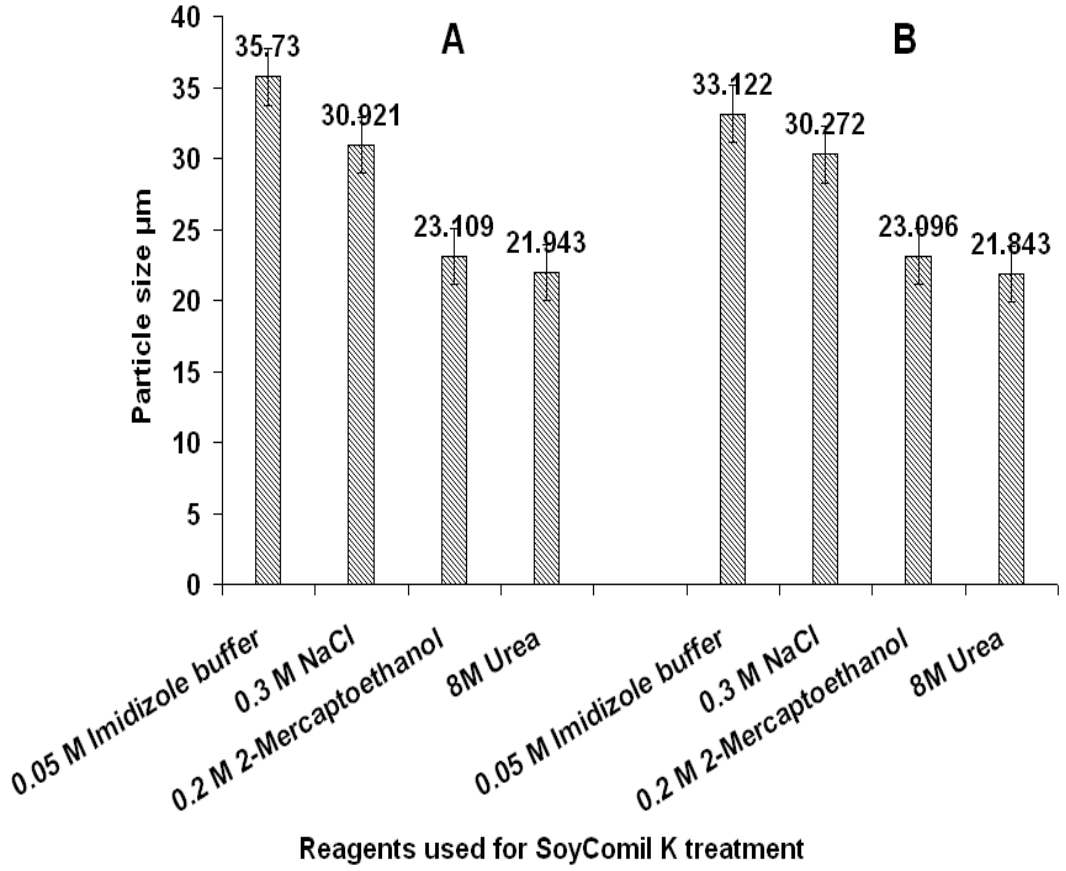

Figure 3.15 Particle size profile of heat treated 6% SoyComil K pH 9 dissolved in different buffers (reduction in particle size that should reflect the molecular forces contributing to maintenance of protein structure)

Where A: Treatment at room temperature and B: treatment at 80°C for 10min

3.3.2.6.2 Effect of pH, temperature and protein concentration on SH groups of SoyComil K

The results of heating SoyComil K at different concentrations (6, 10, and 18% w/v) and different pH (4.6, 6.5 and 9) at different temperatures (room temperature, 50 and 80°C) for 10 min (Figure 3.16) show the highest SH- group concentration was obtained at SoyComil K concentration 6%, 80°C and pH 9 was 0.68 μ mol/gm. However, the lowest SH group concentration was at pH 4.6, SoyComil K concentration 18% and room temperature (0.136 μ mol/gm). Under all experimental conditions, the highest SH group was obtained at 6% where 18% protein showed the lowest value of SH groups.

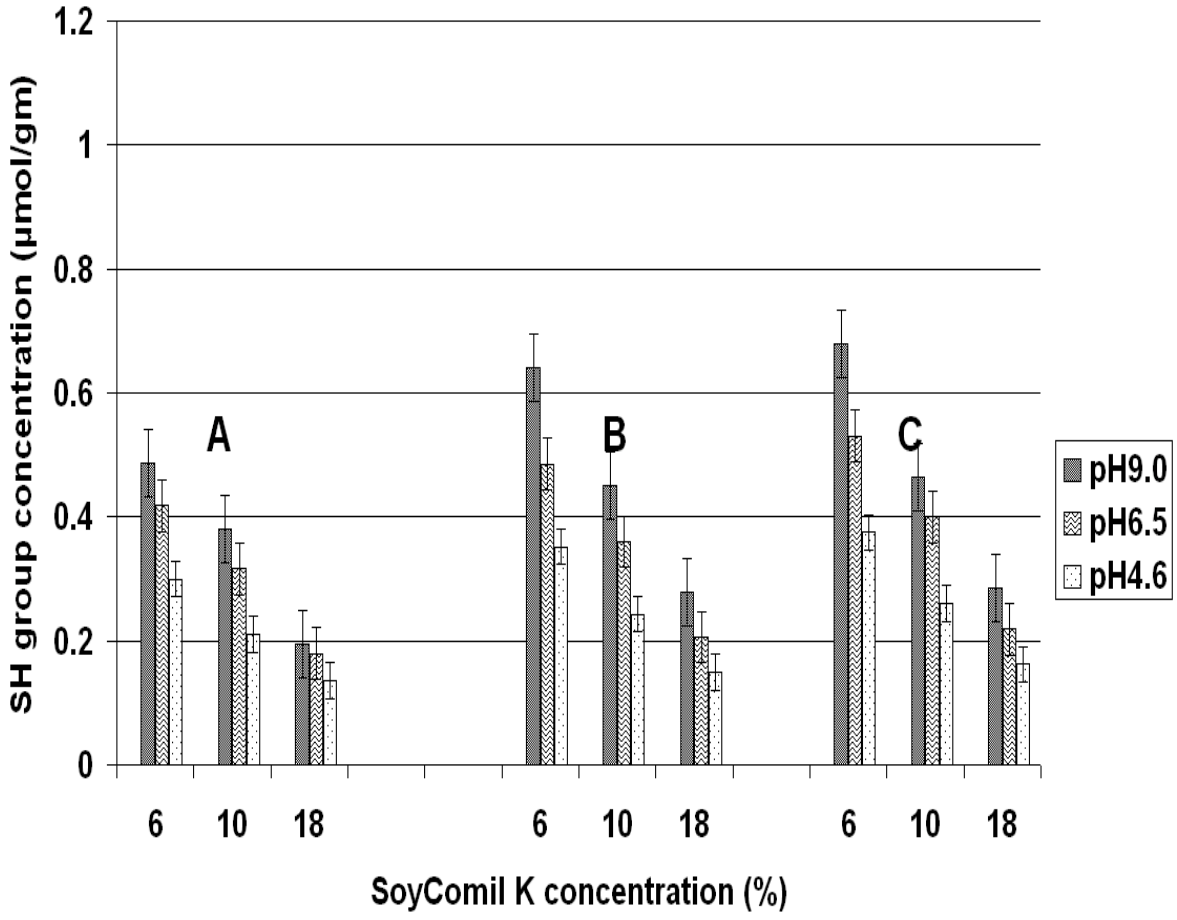

Figure 3.16 Effect of pH, protein concentration and heat treatment on SH groups of SoyComil K

3.3.2.7 Relationship between solubility and hydrophobicity in SoyComil K

Different solubilities of SoyComil K (highest and lowest) were chosen at the same pH and temperature to show the relation between hydrophobicity and solubility. Hydrophobicity data in Table 3.1 indicate that the highest increase in hydrophobicity (300.12) was at pH 9, 80°C, solubility 8.42% and concentration 6%, whereas the lowest increase in hydrophobicity (78.60) was at pH 4.6, room temperature, solubility 0.11% and concentration 18%. Table 3.1 shows that hydrophobicity of SoyComil K at pH6.5 increased from 97.64 to 262.1. As the solubility increased, the hydrophobicity increased under all experimental conditions.

Table 3.1 Effect of pH, protein concentration and heat treatment on SoyComil K solubility and hydrophobicity

pH	Temperature	Concentration			
		6% (w/v)		18% (w/v)	
		%Solubility	Hydrophobicity	%Solubility	Hydrophobicity
pH9.0	RT	2.21	220	0.65	116.24
	50°C	3.61	255.30	0.90	142.10
	80°C	8.42	300.12	1.35	159.30
pH6.5	RT	0.78	188.32	0.36	97.64
	50°C	1.73	242.62	0.56	124.30
	80°C	2.47	262.10	0.73	149.60
pH4.6	RT	0.31	157.39	0.11	78.60
	50°C	0.40	229.01	0.19	109.07
	80°C	0.63	246.30	0.29	130.20

Where RT: room temperature (22± 3°C)

3.3.3 Properties of SoyComil K emulsions

In many cases the application of soy proteins is limited due to incompatibility between their solubility and other properties such as emulsifying activity (Wang *et al*., 2008). To achieve desirable properties, many physical, chemical and enzymatic modifications have been applied to soy protein.

3.3.3.1 Effect of different treatments on oil droplet size of SoyComil K emulsions

The study was undertaken to determine the effect of different treatments of SoyComil K on its emulsification ability. Due to the poor behaviour of SoyComil K as emulsifier, which is expressed in the large oil droplet size for untreated SoyComil K (unheated control) corresponding to D(3,2). D(3,2) is defined as the diameter of a sphere that has the same volume/surface area ratio as a particle of interest or the ratio of the third to second moment of the probability density function (Liu et al., 2007; Pacek *et al.*, 1998; Downs and Sarv, 2003; chapter 2 section 2.2.8.1.1). SoyComil K (6%), pH7 was pre-treated by enzymes (proteinase K and α-amylase), or mixed with 10% glucose and heat treated (100°C for 10 min), as described in Chapter 2, section 2.2.8.1.1. The average droplet size distribution D(3,2) of SoyComil K emulsions was measured immediately after emulsion formation. Results of average droplet size D(3,2) are shown smallest drop;et size was obtained with emulsion contain glucose in unheated and heated emulsions, which are 30.05 μm and 6.21 μm respectively (Figure 3.17). However, the largest droplet size was obtained with non-heated and heated control (SoyComil K only), which are 39.82 μm and 7.0 μm respectively. Over all highest droplets size obtained with non-heated (SoyComil K only), and smallest was with heat treated SoyComil K. There is no significant difference in oil droplet size between emulsion treated by proteinase K and emulsion contain glucose.

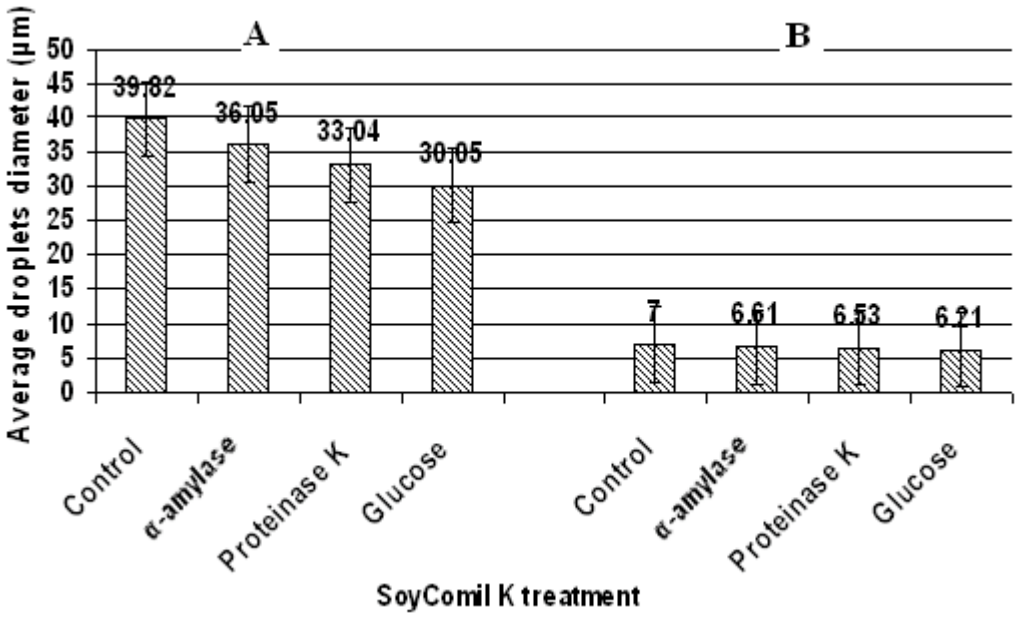

Figure 3.17 Effect of different treatments of SoyComil K on the average droplet size of SoyComil K emulsions.

Where A: are emulsions of non-heated 6% SoyComil K

B: are emulsions of 6% SoyComil K heated to 100°C for 10 min. .

3.3.3.2 Effect of different treatments on emulsion stability of SoyComil K

Emulsion stability caused by proteins can be used to define how these proteins can be added to existing foods and how they can replace more expensive proteins traditionally used. The stabilizing effect of proteins in emulsions results from the protective barrier they form around fat droplets, which further prevents their coalescence (Kinsella, 1979). Long –term stability of emulsions depends basically on the thickness and strength of adsorbed protein films at the oil- water interface (Zayas and Lin, 1989). Heat treatment, enzyme hydrolysis (proteinase, α-amylase) and heat treatment in the presence of glucose were applied to improve the emulsification properties of SoyComil K. The results in this section show that heat treatment of SoyComil K before homogenization resulted in emulsions with decreased droplet size and enhanced emulsion stability (Figures 3.18, 3.19, 3.20, 3.21 and 3.22). All emulsions of non-heated SoyComil K showed separation on the first day they were made, even when SoyComil K was treated by enzymes, or mixed with

glucose beforehand (Figures 3.19, 3.20, 3.21 and 3.22). heat treatment of SoyComil K suspensions before homogenization improve emulsion stability and prevent emulsion separation (Figures 3.19, 3.20, 3.21 and 3.22), and this proved in previous study (section 3.3.3.1), where heated suspension had small dropletsize.

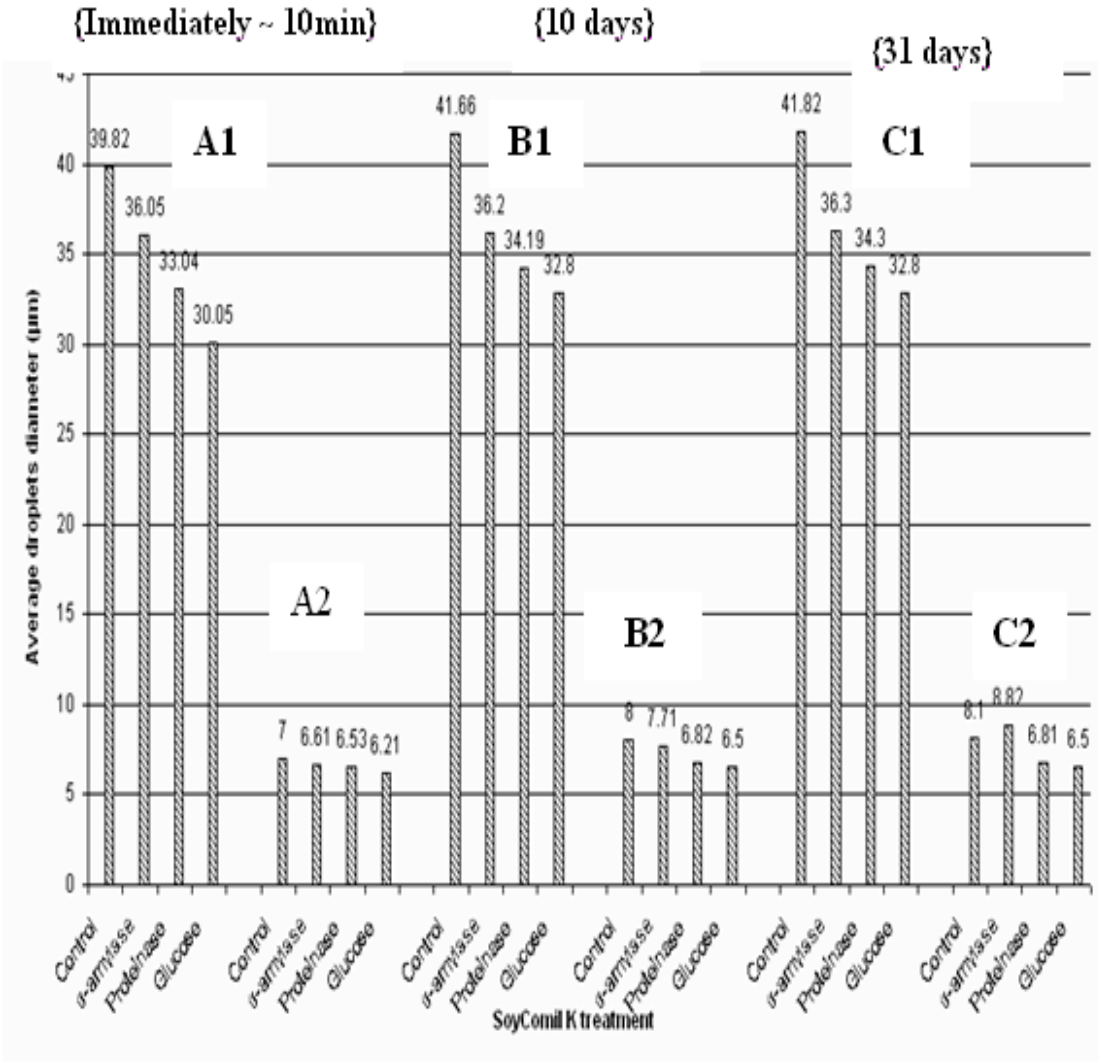

Figure 3.18 Effect of different treatments of SoyComil K on the average droplet size of SoyComil K emulsions at different times.

Where: A1, B1 and C1 are emulsions of non-heated 6% SoyComil K

A2, B2 and C2 are emulsions of 6% SoyComil K with Soycomil treated with enzymes and glucose followed by heating to 100°C for10 min. .

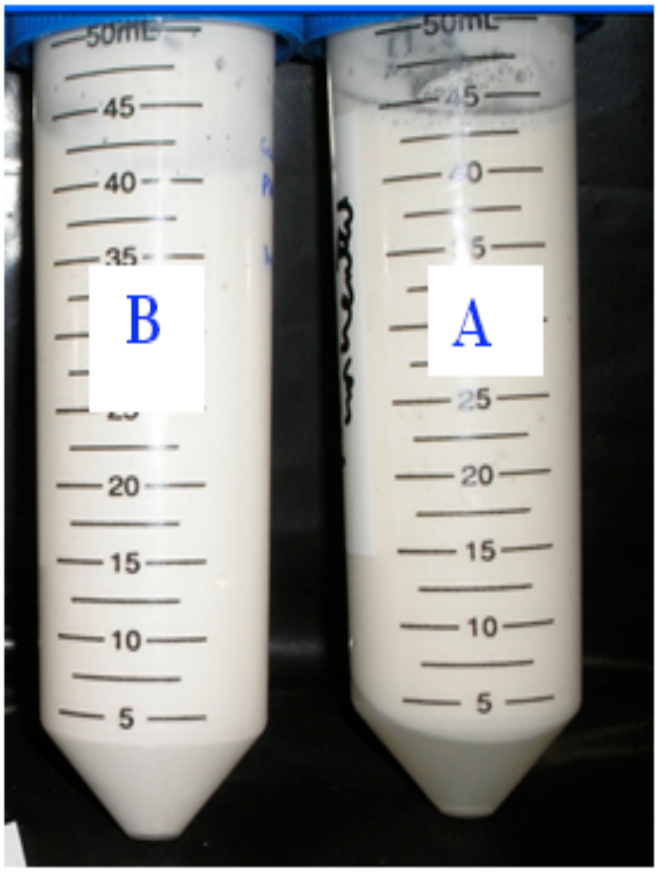

Figure 3.19 Effect of heat treatment of SoyComil K on emulsion stability after 30 days

Where A: 6% SoyComil K, pH7, emulsified with 30% sunflower oil and homogenized at 500 bar at room temperature. (Separation occurred on the 3rd day).
B: 6% SoyComil K, pH11 heated to 100°C for 10 min, emulsified with 30% sunflower oil at pH7 and homogenized at 500bar at room temperature**.**

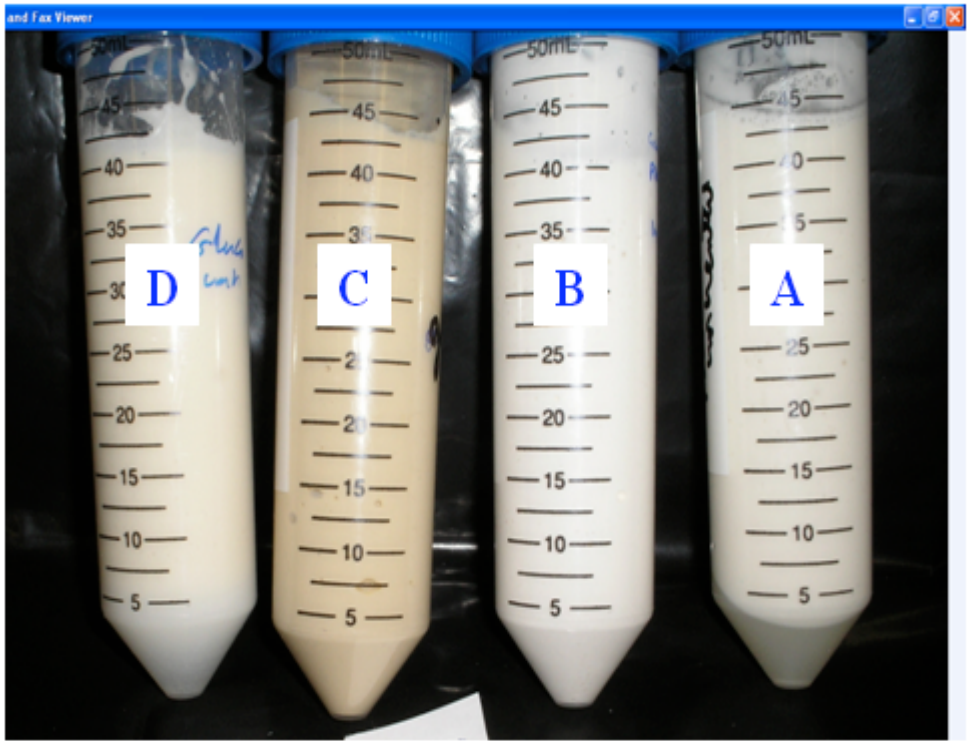

Figure 3.20 Effect of heat treatment of SoyComil K in the presence of glucose on emulsion stability after 30 days

Where **A**: 6% SoyComil K (pH 7), emulsified with 30% sunflower oil and homogenized at 500bar at room temperature. (Separation occurred on the 3rd day).

B: 6% SoyComil K, pH11 heated to 100°C for 10 min, emulsified with 30% sunflower oil at pH7 and homogenized at 500bar at room temperature (22± 3°C).

C: 6% SoyComil K mixed with 10% glucose (pH11 heated to 100°C for10 min) then adjust pH7 and emulsified with 30% sunflower oil and homogenized at 500 bar at room temperature.

D: 6% SoyComil K mixed with 10% glucose, pH7, emulsified with 30% sunflower oil then homogenized at 500bar at room temperature and (separation occurred on the 3rd day).

Figure 3.21 Effect of α-amylase treatment followed by heat treatment of SoyComil K on emulsion stability after 30 days

Where **A**: 6% SoyComil K, pH 7, emulsified with 30% sunflower oil and homogenized at 500bar at room temperature. (separation occurred on 3rd day).

B: 6% SoyComil K, pH11 heated to 100°C for 10 min, emulsified with 30% sunflower oil at pH7 and homogenized at 500bar at room temperature.

C: 6% SoyComil K treated by α-amylase for 6h, followed by adjustment to pH11 and heated to 100°C for 10 min, then re-adjustment of pH7 and emulsified with 30% sunflower oil then homogenized at 500 bar at room temperature.

D: 6% SoyComil K treated by α-amylase for 6h then adjust pH7 and emulsified with 30% sunflower oil at room temperature then homogenized at 500 bar at room temperature.

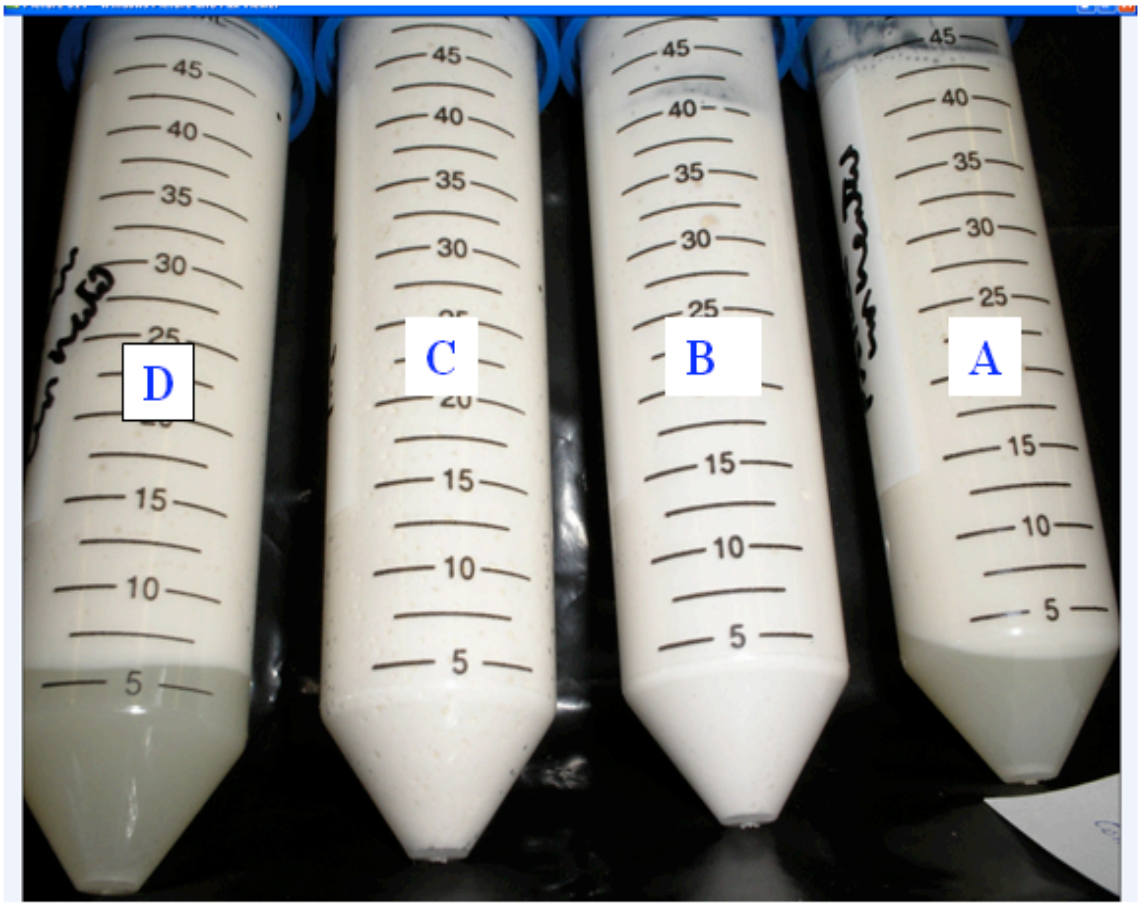

Figure 3.22 Effect of protease treatment followed by heat treatment of SoyComil K on emulsion stability after 30 days

Where **A**: 6% SoyComil K, pH 7, emulsified with 30% sunflower oil and homogenized at 500bar at room temperature. (separation occurred on 3rd day).

B: 6% SoyComil K (heated to 100°C for 10 min at pH11) then adjust pH7 and emulsified with 30% sunflower oil then homogenized at 500bar at room temperature.

C: 6% SoyComil K treated by protease for 6h, followed by adjustment to pH11 and heated to 100°C for 10 min, then re-adjustment of pH 7 and emulsified with 30% sunflower oil then homogenized at 500 bar at room temperature.

D: 6% SoyComil K treated by protease for 6h then adjust pH7 and emulsified with 30% sunflower oil at room temperature then homogenized at 500 bar at room temperature.

3.3.3.3 Rheological measurements

3.3.3.3.1 Effect of different treatments of SoyComil K on emulsion viscosity

Viscosity of SoyComil K emulsions was measured after homogenization at 500bar at room temperature and after ageing for less than 20 min. Figures 3.23, 3.24, 3.25 and 3.26 show typical curves of viscosity as a function of shear rate of emulsions prepared with SoyComil K, which had undergone different treatments (heat treatment, enzymatic hydrolysis " proteinase and α-amylase", and mixed with 10%glucose). It is clear that the rheological response of the emulsion was highly dependent on SoyComil K treatment. Emulsions of heated SoyComil K solution had under all circumstances a higher viscosity than unheated emulsions. There was a linear relationship between shear rate and viscosity.

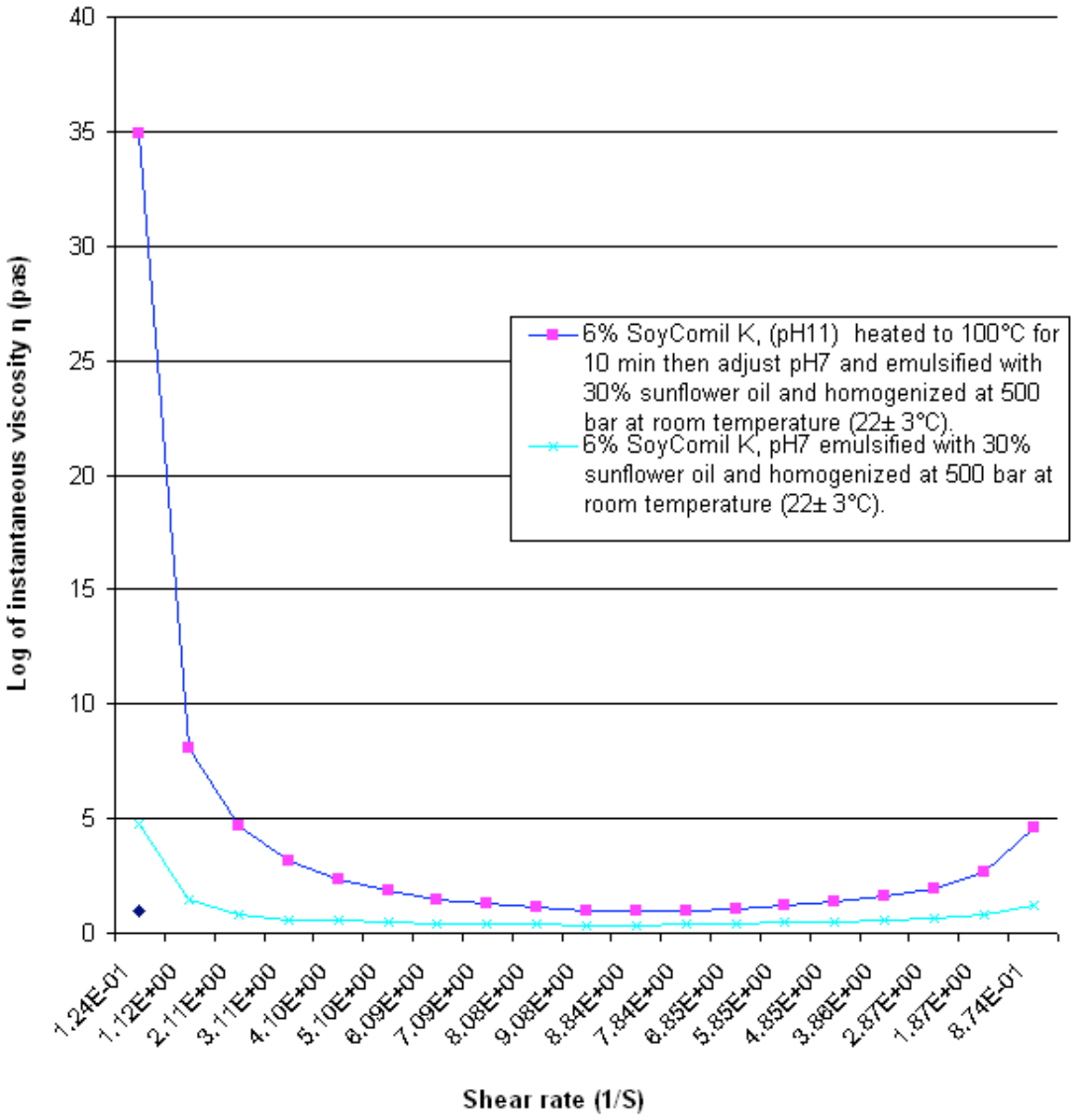

Figure 3.23 Effect of heat treatment of SoyComil K on emulsion viscosity

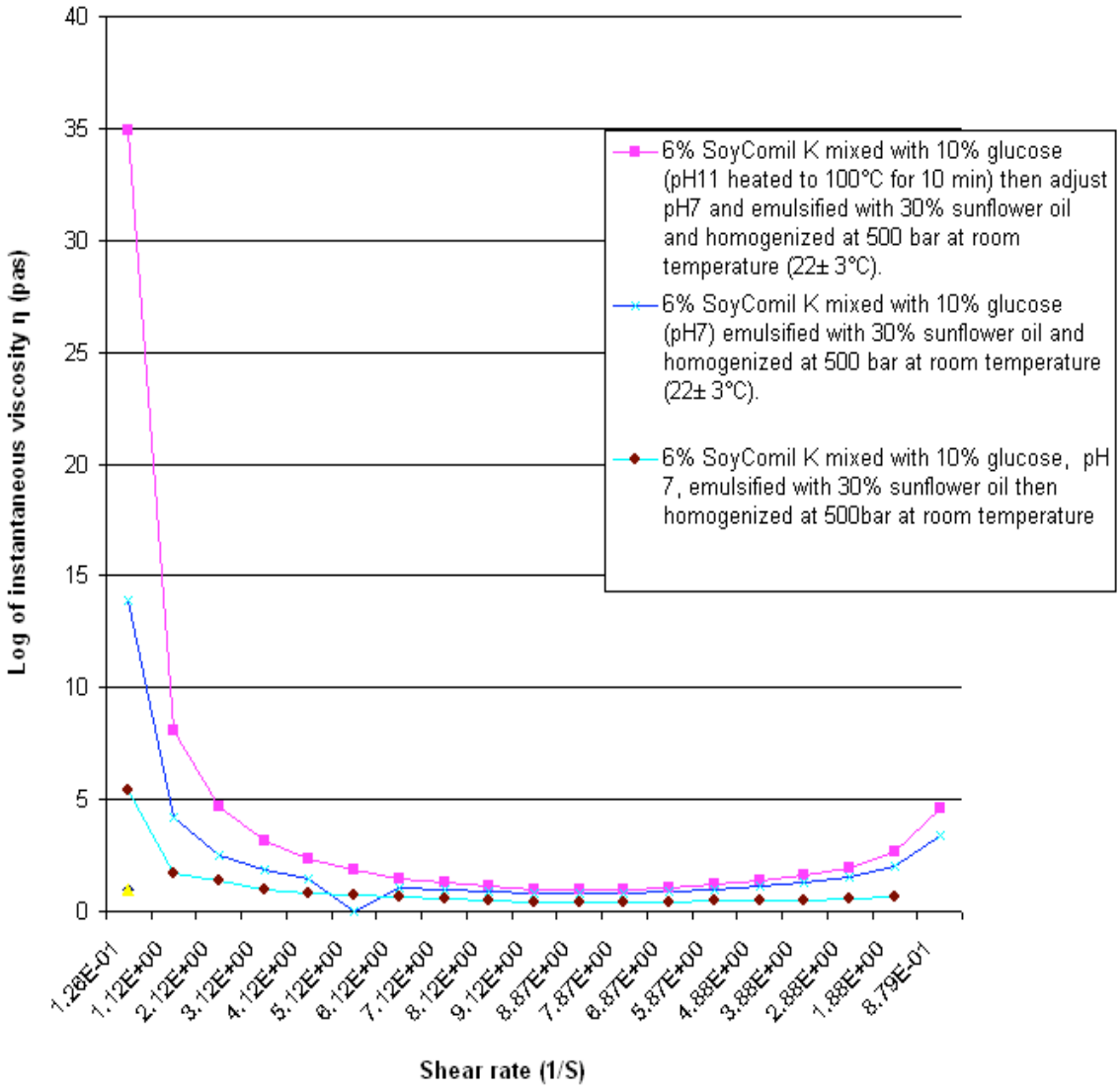

Figure 3.24 Effect of heat treatment of SoyComil K in the presence and absence of glucose on emulsion viscosity

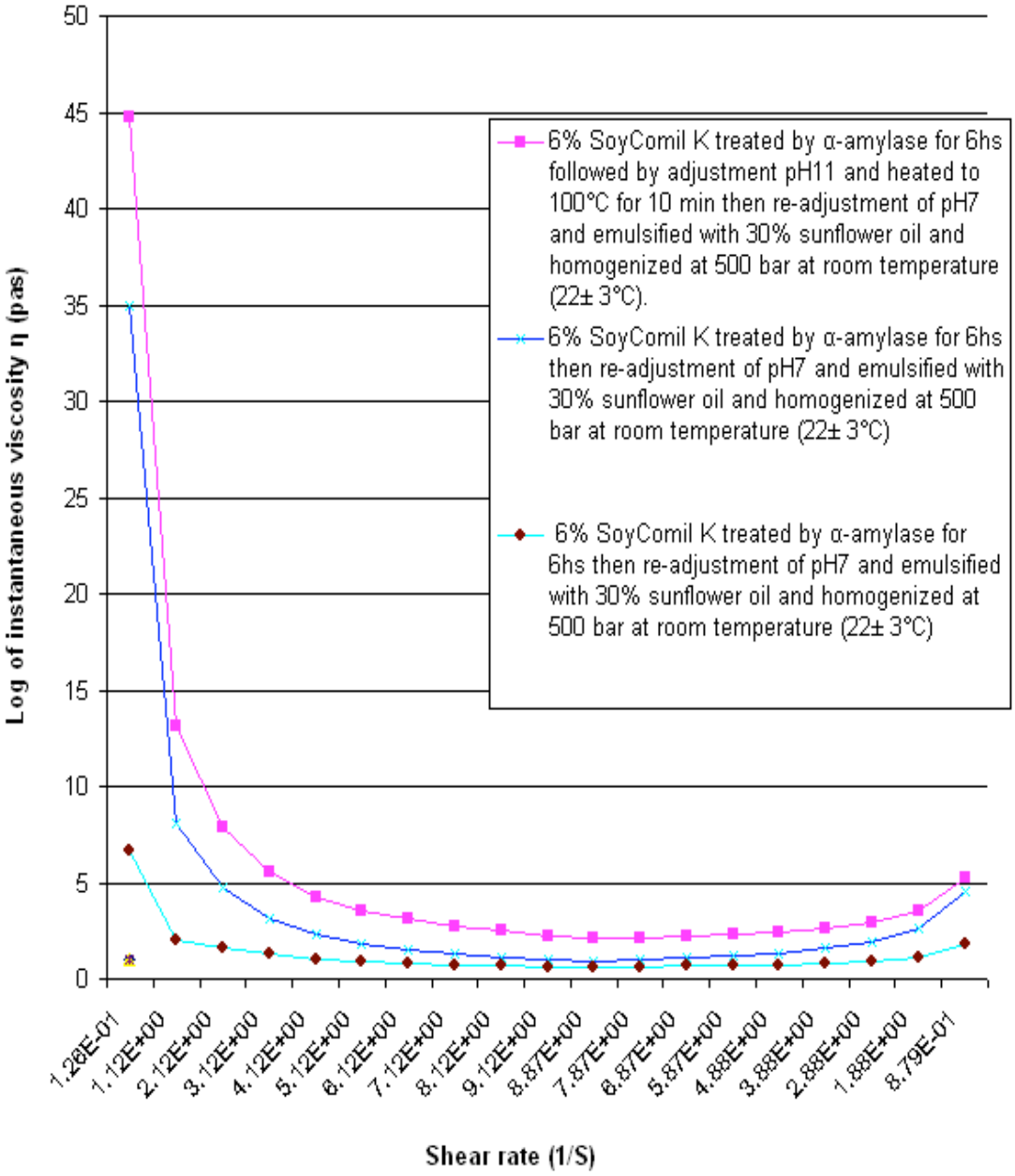

Figure 3.25 Effect of α-amylase treatment followed by heat treatment of SoyComil K on emulsion viscosity

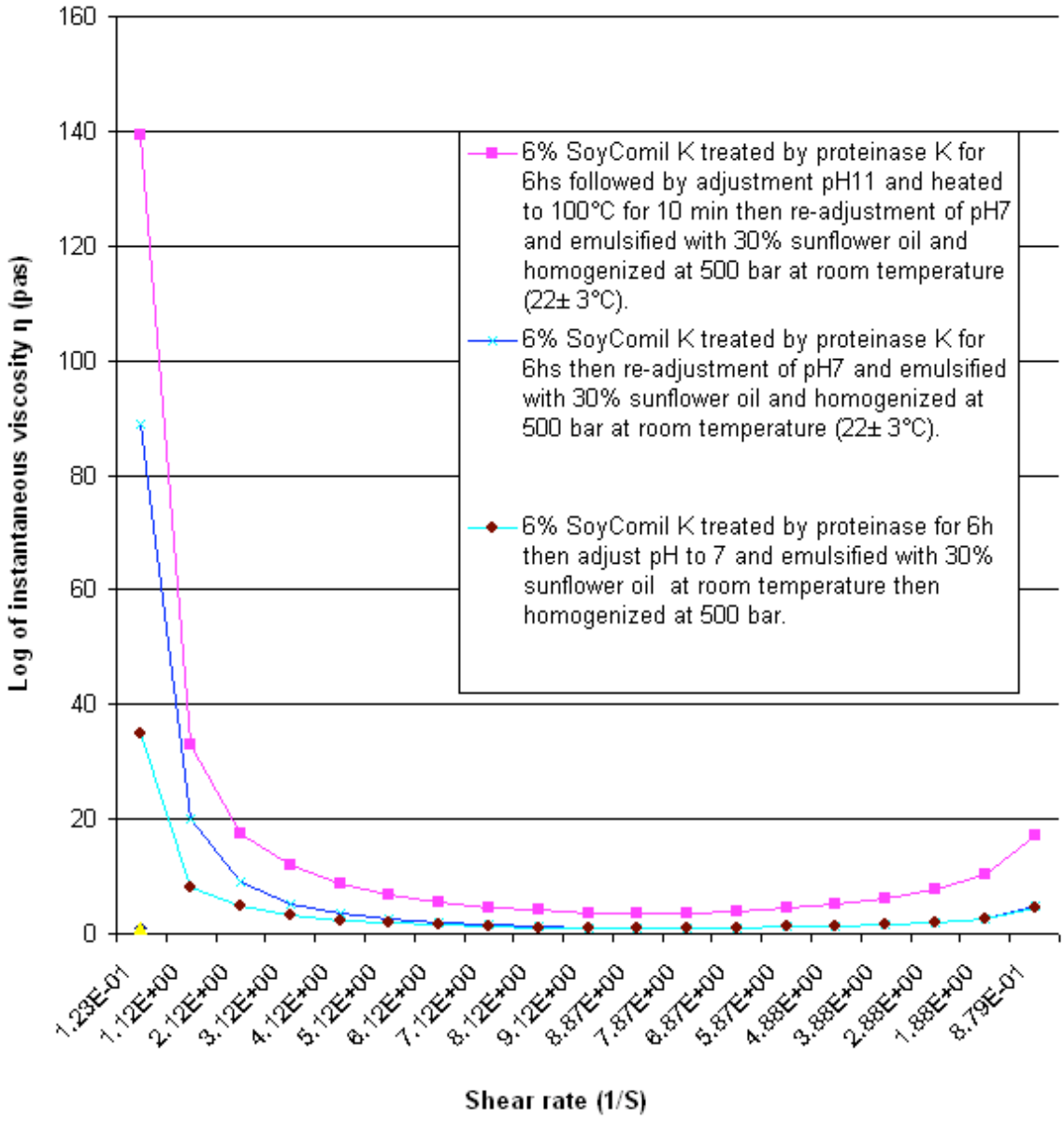

Figure 3.26 Effect of proteinase treatment followed by heat treatment of SoyComil K on emulsion viscosity

3.3.3.4 Confocal laser scanning microscopy of SoyComil K emulsions

The largest benefit of confocal laser scanning microscopy (CLSM) images was to elucidate the sizes and locations of all the droplets in the image. It was then possible to examine detailed structural information at a fixed point to determine whether any changes in microstructure could be detected that might explain the above rheological changes. Figures 3.27, 3.28, 3.29, 3.30, 3.31, 3.32, 3.33 and 3.34 show the images of emulsions (of

unheated and heated SoyComil K treated by enzymes “proteinase K and α-amylase”, or mixed with 10 % glucose). The images show that the droplets size of emulsions of non-heated SoyComil K looks larger than emulsions of heat-treated SoyComil K. In the latter emulsions spherical aggregates could be visualized, which were not observed in the former emulsions. This provides qualitative evidence of structural changes in emulsion formed by heat-treated SoyComil K.

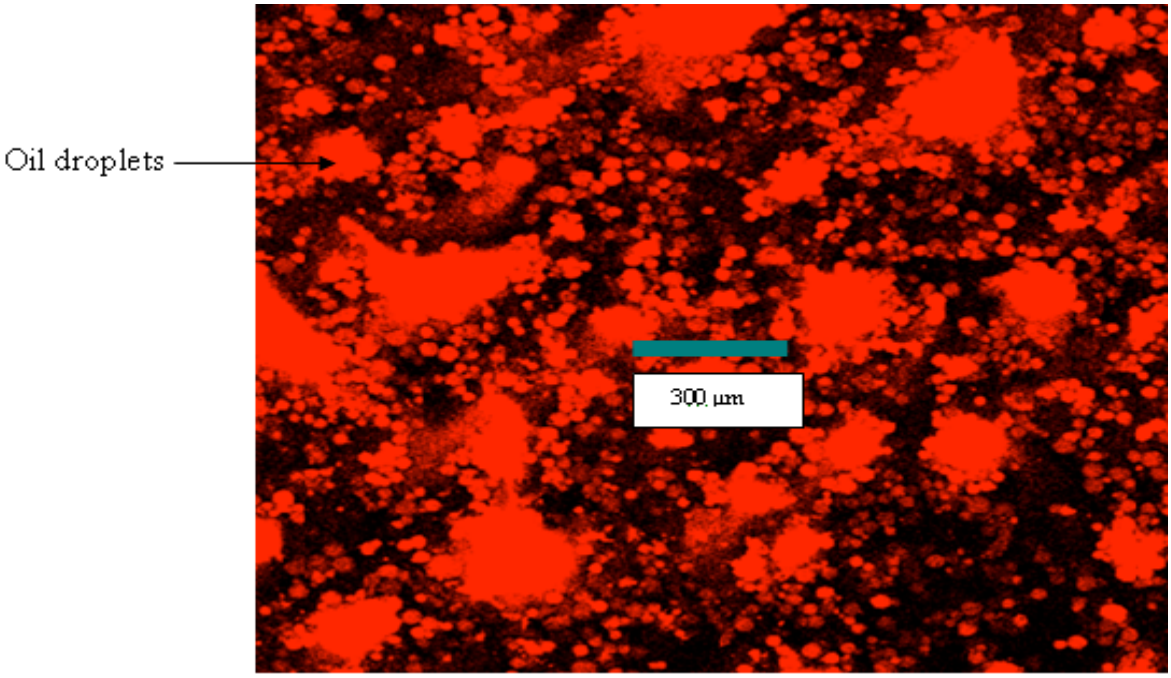

Figure 3.27. SoyComil K (6%), pH 7, emulsified with 30% sunflower oil and homogenized at 500bar at room temperature (22± 3°C).

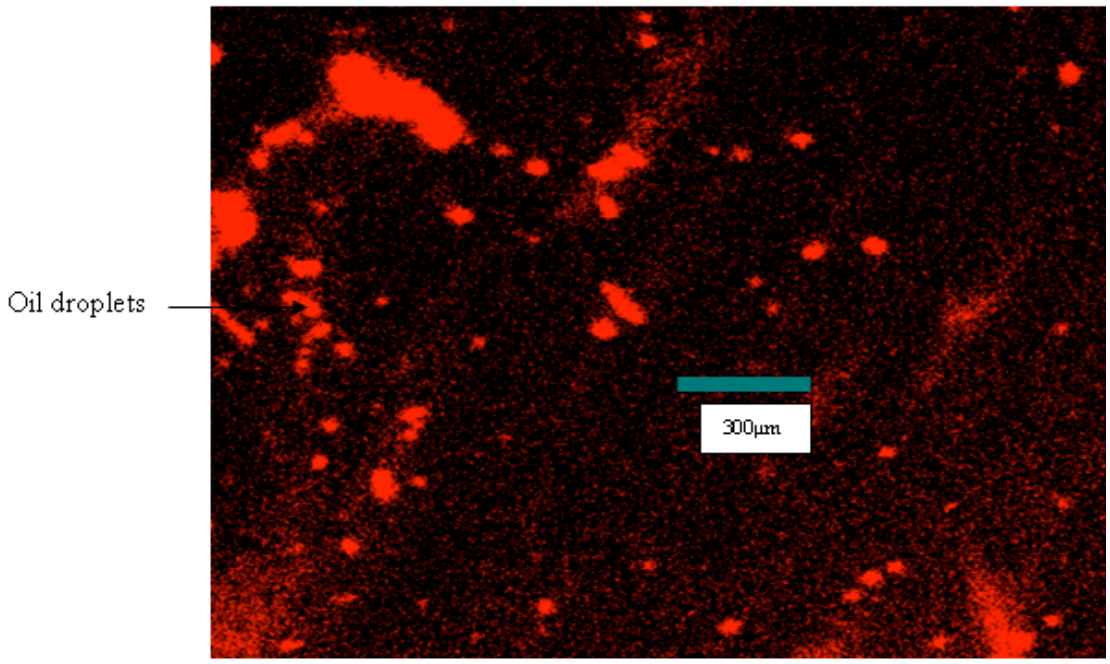

Figure 3.28 SoyComil K (6%), pH11 heated to 100°C for 10 min, emulsified with 30% sunflower oil at pH7 and homogenized at 500bar at room temperature (22± 3°C).

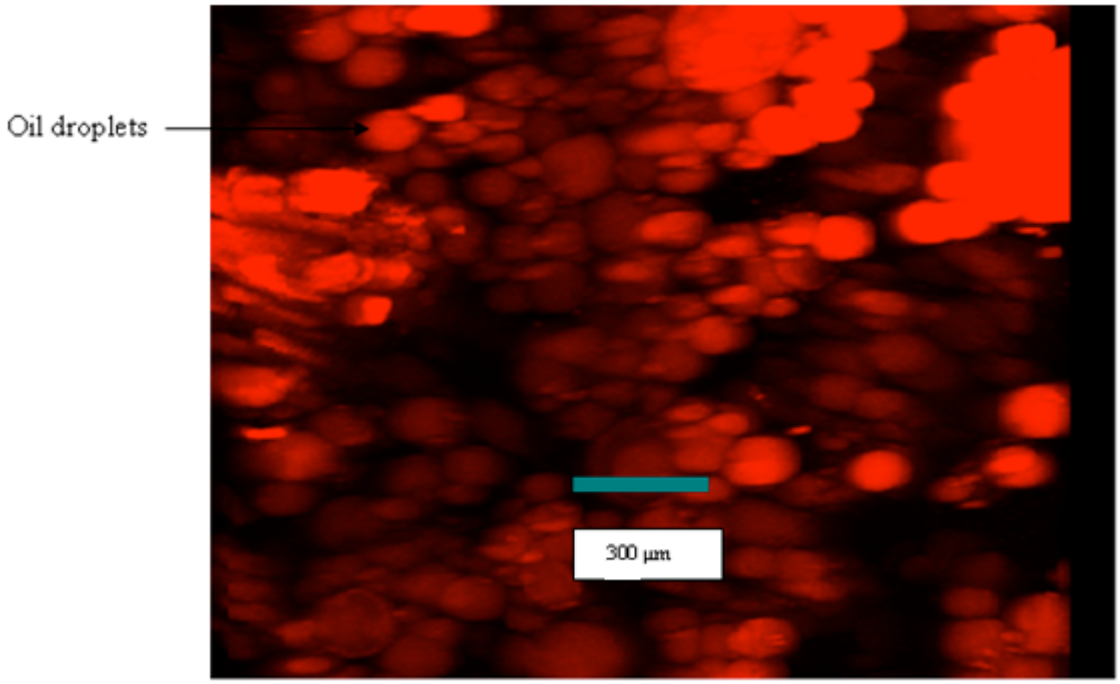

Figure 3.29 SoyComil K (6%) mixed with 10% glucose, pH 7, emulsified with 30% sunflower oil then homogenized at 500bar at room temperature (22± 3°C).

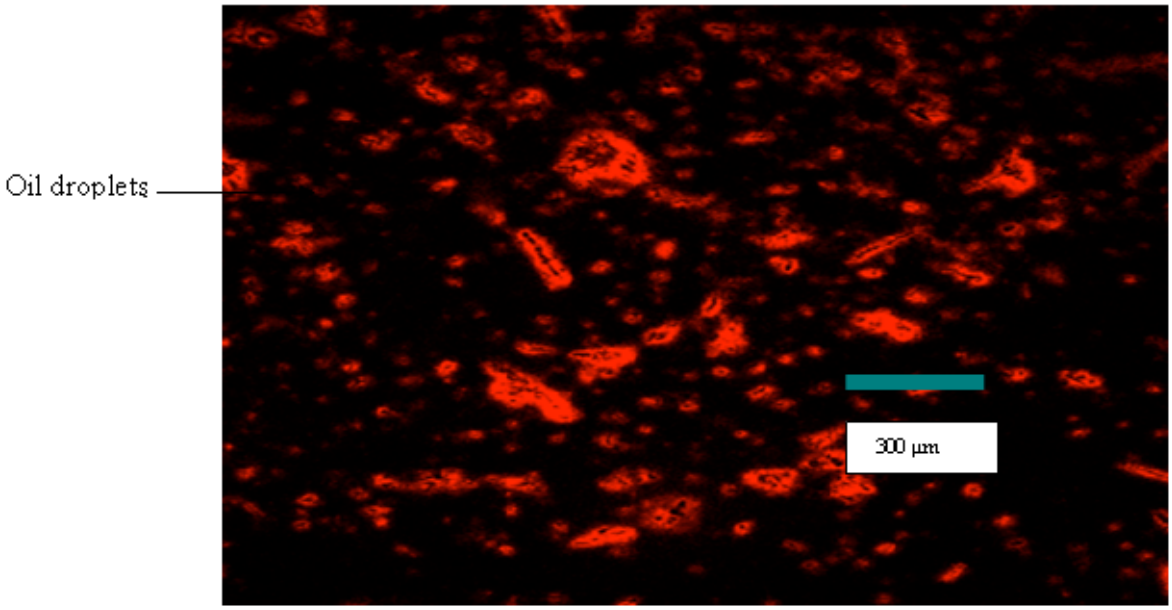

Figure 3.30 SoyComil K (6%) mixed with 10% glucose (heated to 100°C for10 min pH11) then adjust pH7 and emulsified with 30% sunflower oil and homogenized at 500 bar at room temperature (22± 3°C).

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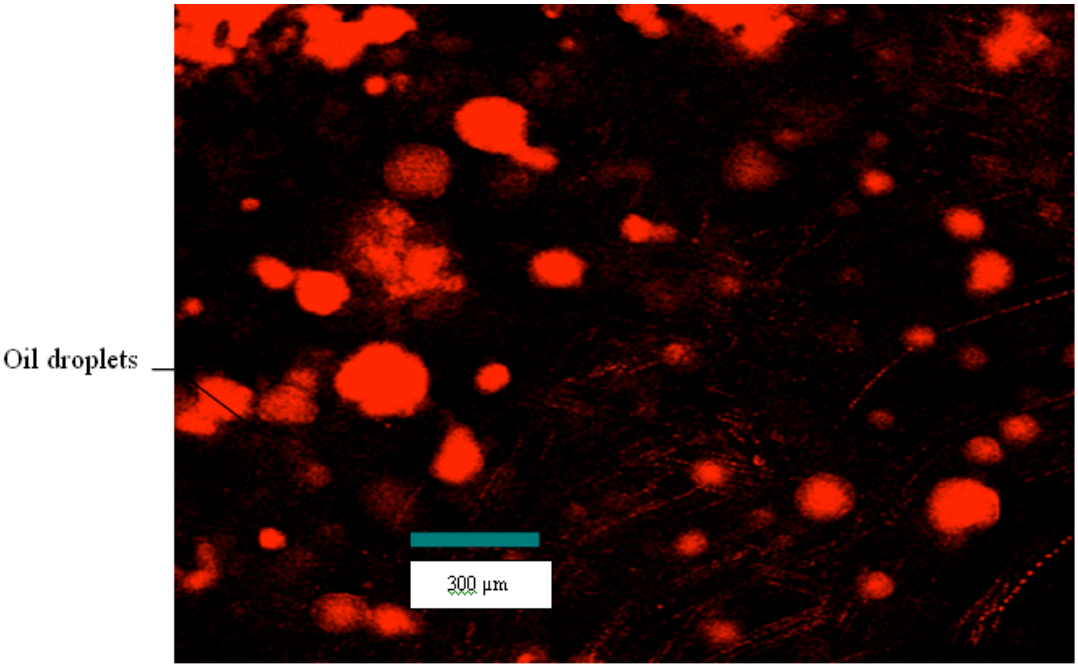

Figure 3.31 SoyComil K (6%) K treated by α-amylase for 6hs then adjusts pH7 and emulsified with 30% sunflower oil at room temperature then homogenized at 500 bar at room temperature (22± 3°C).

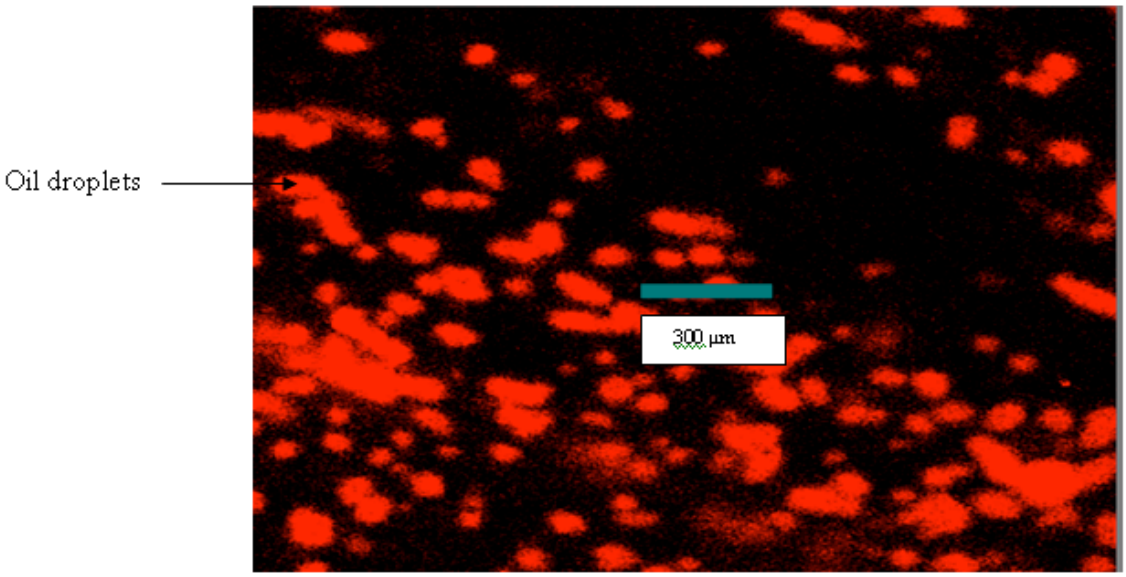

Figure 3.32 SoyComil K (6%) treated by α-amylase for 6hs, followed by adjustment to pH11 and heated to 100°C for10 min, then re-adjustment of pH7 and emulsified with 30% sunflower oil then homogenized at 500 bar at room temperature (22± 3°C).

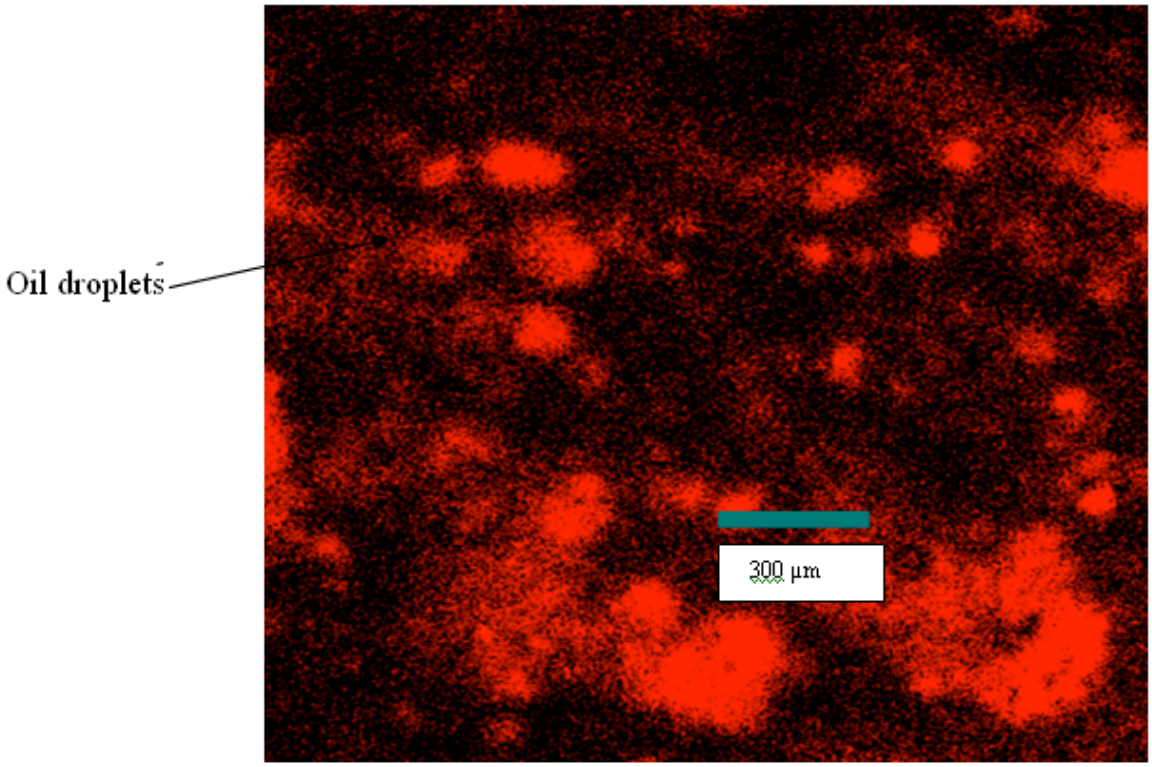

Figure 3.33 SoyComil K (6%) treated by proteinase for 6hs then adjust pH7 and emulsified with 30% sunflower oil at room temperature then homogenized at 500 bar at room temperature (22± 3°C).

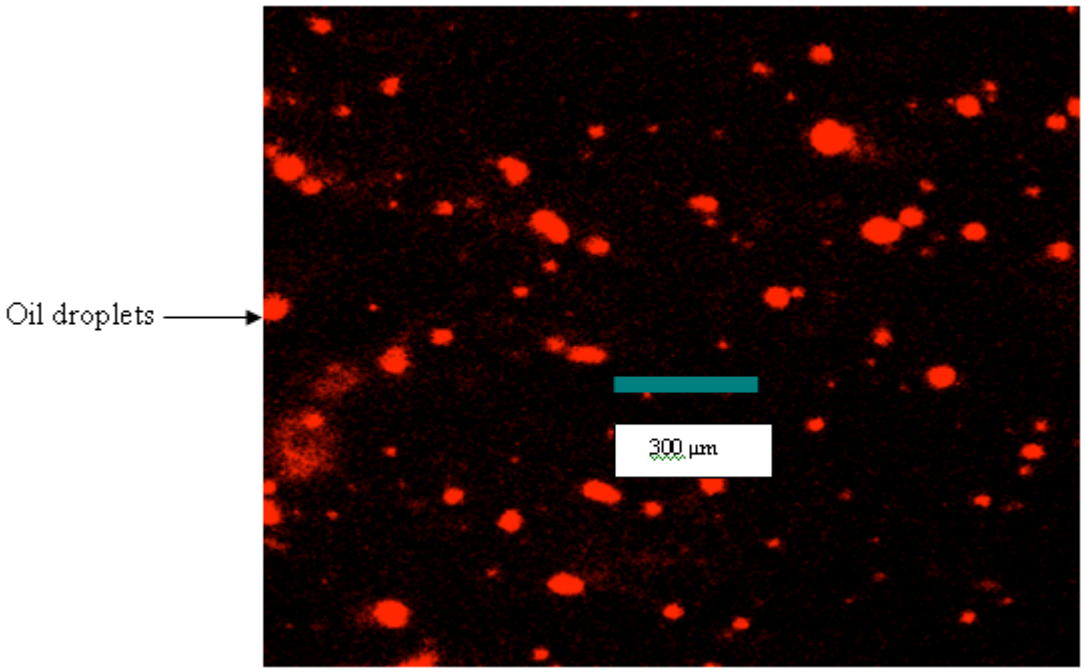

Figure 3.34 SoyComil K (6%) treated by proteinase for 6hs, followed by adjustment to pH11 and heated to 100°C for10 min, then re-adjustment of pH7 and emulsified with 30% sunflower oil then homogenized at 500 bar at room temperature (22± 3°C).

3.4 Discussion

3.4.1 Physicochemical properties of Soycomil K

3.4.1.1 SDS-PAGE electrophoresis

Reducing and non-reducing SDS-PAGE of supernatants and pellets of SoyComil K (pH9) was carried out to identify the soluble and insoluble fractions (Figure 3.1). To elucidate the factors that determine solubility of soy protein, SDS-PAGE of SoyComil K, soy Arcon ® SJ and SPI at pH7 (Figures 3.2 and 3.3) were studied. The major proteins of soy protein are glycinin (11S) and β- conglycinin(7S). These two proteins account for 70% of total soy protein (Abtahi and Aminlari, 1997). In this study, the electrophoretic patterns show 7S and 11S globulin subunits for the all samples. The results suggest the formation of complexes between the 7S and 11S subunits, which were induced by heat treatment (Yamagishi et al., 1983). The major constituent bands in all samples were situated between 6-64 KDa. The intensity of β- conglycinin subunits were more than glycinin under non-reducing conditions. Because glycinin subunits are associated through, intermolecular disulfide bridges which are buried in the interior part of the molecules, (Romagnolo et al., 1990) its dissociation by SDS under non-reducing conditions was very little compared to *β*- conglycinin. The lack of high intensity bands in the pellet of SoyComil K under non-reducing conditions may be due to aggregation of protein at the origin of lanes, which was not dissociated by SDS (Yamagishi et al., 1983).

The intensity of bands was increased under reducing conditions, because 2-mercaptoethanol cleaved the disulfide bonds of large protein aggregates. Lanes of 6% SoyComil K (Figure 3.1 lanes 1, 2, 6 and 7) showed bands with lower intensity than 1% of SoyComil K (Figure 3.1 lanes 3, 4, 8, 9 and 10). It is possible I hypothesize that when SoyComil K is present at high enough concentration to form aggregates, it does not enter the gel, but stay at the origin of lanes, while at lower concentrations various types of soluble complexes may form (Roesch, and Corredig, 2005). SoyComil K shows less bands with lower intensity than soy Arcon ® SJ and SPI, which could be due to its poor solubility.

In summary it can be said that the intensity and number of bands under reducing conditions are more than those under non- reducing conditions, due to the ability of 2-mercaptoethanol to cleave disulfide bonds thereby facilitating the solubilisation of large protein aggregates

held together by disulfide bridges, which cannot be achieved by SDS (Yamagishi *et al*., 1983).

3.4.1.2 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) can reveal structural and conformational changes of proteins during heat treatment. The denaturation temperature (peak of denaturation) indicates the protein's thermo-stability, while Δ*H* (denaturation enthalpy) is an indication of hydrophobic/hydrophilic interactions and compactness of proteins (Hua *et al*., 2005). The results show that SoyComil K used in this study had a denaturation degree of 27.99% compared to native laboratory prepared soy protein concentrate (Figure 3.4 and 3.5). The degree of protein denaturation was calculated by dividing Δ*H* associated with the denaturation peak of glycinin of pretreated protein (Soycomil) to that of an untreated SPC (native) protein (Δ*H* treated/ Δ*H* native) (Sorgentini *et al*., 1995; Renkema, 2001). The difference between SoyComil K and native soy protein concentrate was due to partial denaturation of SoyComil K, as a result of its isolation procedure. The appearance of exothermic peaks of *β*-conglycinin and glycinin at 74.72 °C and 102.79 °C respectively, indicates that SoyComil K was not completely denaturated during manufacturing.

The denaturation temperature of glycinin of SoyComil K is higher than native laboratory prepared soy protein concentrate. This could that be due to the different genetic variants of glycinin which display different thermostability (Lakemond *et al*., 2002a). On the other hand, it could also be due to the formation of a more stable conformation of glycinin during the isolation procedure of SoyComil K.

The denaturation temperature of glycinin was significantly higher (80 ºC) than that of *β*-conglycinin (76ºC) in both native laboratory prepared soy protein and SoyComil K, confirming the findings of Renkema and Van Vliet, (2002). The data show broad exothermic transitions in the DSC thermogram. This could be explained by the fact that both the *β*- conglycinin and glycinin fractions are composed of several genetic variants, which have different thermal stabilities (Maruyama *et al*., 1998).

3.4.2 Factors influencing Soycomil K solubility

3.4.2.1 Effect of pH, temperature and protein concentrations on SoyComil K solubility

Protein structure can be modified by different treatments to improve specific functional properties, heating being one of the more frequently methods used for that purpose (Sorgentini *et al.*, 1995). In this study, the effect of heat treatment under different conditions (temperature, protein concentration, and pH) on functional properties such as solubility was investigated (figure3.6). Solubility is the amount of a solute that can be dissolved in solvent. Solubility refers to proteins that are not aggregated or are present in aggregates too small to sediment upon centrifugation (Renkema *et al*., 2000). The main problem of SoyComil K quality is its insolubility and resistance to heat. This thermal stability behaviour is attributed to its structure. In this study, it was observed that solubility dramatically increased as the pH increased. The solubility was at its lowest at pH 4.6, which is the isoelectric point of soy protein, where the electrostatic repulsive forces are insufficient to prevent extensive aggregation (Malhotra and Coupland, 2004). Solubility increased progressively the further the pH was from the isoelecric point and solubility was at its highest at pH 9. This was due to increased electrostatic repulsive forces between particles, which weakened the hydrophobic associations and resulted in increased solubility (Mo et al., 2006). Under alkaline conditions, some hydrogen bonds in protein molecules are broken, causing it to assume a configuration somewhat more open than its original configuration, causing many buried peptide groups and side chains to become exposed to solvent (Mirsky and Pauling, 1936). It was observed that the solubility of all samples were pH dependent. It was also observed that non-heated SoyComil K had lower solubility than the heated version at all studied pH, the increased solubility corresponding to increased temperature could be due to dissociation of aggregates (oligomers) into subunits of smaller molecular weight (Shimad and Cheftel. 1988 and Rangavajhyala et al., 1997) as well as partial denaturation of monomers. Utsumi *et al.*, (1984) has shown that thermal treatment of soy protein induces formation of soluble macro complexes between the subunits of 7S globulin and basic subunit of 11S globulin. As the concentration of SoyComil K increased, its solubility decreased, probably caused by increased protein-protein interaction, causing an increase in the burial of charged groups (Pace *et al*., 2004; Wagner *et al.*, 2000 and Sorgentini *et al.*, 1995). Lower solubility at higher protein concentration, may also be

caused by intermolecular S-S bonds resulting in while at lower concentrations various types of soluble complexes may form (Roesch, and Corredig, 2005).

3.4.2.2 Effects of pH, temperature and concentration on turbidity of SoyComil K

Turbidity was measured by light scattering, where sample in buffer will cause an incident light beam to be scattered in directions other than a straight line through a sample.The highest increase in turbidity was at pH 4.6, whereas the lowest increase in turbidity was at pH 9 (Figure. 3.7). Under all experimental conditions, the highest turbidity was obtained with 18% protein, whereas 6% protein showed the lowest turbidity. Non-heated SoyComil K had higher turbidity than heat-treated (Figure. 3.7).

Thermal treatment of SoyComil K decreased turbidity, probably due to dissociation of aggregates, leading to increased solubility (Utsumi et al., 1984). Figure 3.7 shows that turbidity increased as pH decreased and the highest turbidity was obtained at the iso-electric point, pH 4.6. The decrease in turbidity observed with increase in pH could be due to increased dissociation of aggregates (Sripad and Rao, 1987), and increase of electrostatic repulsion (Alting *et al*., 2002). As the concentration of SoyComil K increased the turbidity increased, due to increased association of aggregation by protein-protein hydrophobic interactions (Sorgentini et al., 1995 and Renkema et al., 2002).

3.4.2.3 Effect of salt (NaCl) on SoyComil K solubility

Protein solubility has long been known to be very sensitive to salt (Jenkins, 1998). Salts affect in widely different manners the properties of proteins such as their stability and solubility (Arakawa and Timasheff, 1984). The cations and /or anions coming from salts in solution, greatly affects protein solubility (Puppo and Añón, 1998). The effect of salt concentration on protein solubility depends on a salting-in region at low salt concentrations and a salting-out region at high ionic-strength solutions. The salting-in region is a result of favourable electrostatic interactions between the salt ions and the charged residues of the protein. At higher salt concentrations, most salt ions are excluded from the protein's domain due to unfavourable interactions between the salt ions and the hydrophobic residues of the protein, producing salting-out behaviour (Curtis *et al*., 1998). Figure 3.8 shows that SoyComil K solubility increased up to NaCl concentration of 0.4 M (salting-in), and thereafter started to decrease (salting-out). The increase in NaCl content > 0.4M reduced the solubility (salting out), due to the competition between the protein and

saline ions for water molecules. Consequently, the water in the protein neighbourhood was removed increasing the protein-protein interaction, thus leading to aggregation of the protein molecules, followed by precipitation (Ferreira Machado et al., 2007). At low salt concentration solubility of protein increased due to salting in phenomenon by which saline ions interact with groups of opposite protein charges to form a double layer of ionic groups, thereby reducing the electrostatic interaction among the protein molecules and increasing their solvation (Hall, 1996).

3.4.2.4 Effect of some sugars on solubility of SoyComil K

Solubility of SoyComil K with out sugars increased as temperature increased from 70 °C to 100 °C (figures3.9 and 3.100). At higher temperatures, some reactions may take place, such as breakdown of S-S bonds with release of H_2S, release of NH_3 from amide groups, dissociation of subunits and/ or breakdown of these subunits into compounds of small molecular weight, which may be responsible for the enhanced protein solubility (Shimada and Cheftel, 1988). In other hand, the onset denaturation temperature β-conglycinin is 70°C, while that of glycinin is 80°C at neutral pH (Renkema and Van Vliet, 2002). Increased solubility of SoyComil K caused by sugars could be due to hydroxyl groups present in sugar units, which react with hydrophilic amino acid side chains, which contribute to protein-sugar interactions in aqueous solution. These newly formed dipole-dipole interactions could either form a hydrophilic layer around the protein unit and, therefore increase the dispersibility of protein through protein hydration and/or alter the protein intermolecular interactions in such a way that folding and even dissociation may occur (Baier and Mcclements, 2005; Ryan and Brewer, 2005). Highest solubility at 70 °C was obtained by sucrose (3.9), which changes the quality of the aqueous solvent from a thermodynamically-poor to thermodynamically-good one in the presence of sucrose. It is known that low molecular weight sugars can give rather strong “polyfunctional” hydrogen bonds with protein molecules in aqueous medium. . The results indicate that protein-sugar reaction at low temperature (70°C for 30min) is appropriate to increase the solubility of SoyComil K, whereas at high temperature (100°C for 10min), decreased solubility is found 9figure 3.10). This could be due to the Maillard reaction, which caused a decrease in pH due to formation of acidic by-products and an increase covalent protein cross-linking, which would reduce protein solubility (Gu *et al.*, 2008).

3.4.2.4.1 Glycation degree of SoyComil K

Glycation of SoyComil K was investigated by measuring the free amino group availability using the OPA (ortho-phthaldiadehyde) method (Chevalier *et al*, (2001). Figure 3.11 shows decrease in free amino groups caused by all sugars. Decrease in free amino groups could be attributed to glycation or structural modifications of SoyComil K such as protein polymerization, and cross-linking. Sucrose also resulted in glycation although it is not a reducing sugar. In this case it is assumed that there is isomerisation or breakdown to glucose and fructose under the experimental conditions that allows reaction with protein to occur (Jackson and White, 2006). This result seems to be contradictory with Campbell *et al.*, (2008), who found that no glycation occurred with SPI (soy protein isolates) in presence of the non-reducing sugar (sucrose). No development of yellow or brown colour was visually noticeable in any of the samples. It is concluded that these result reflect glycation in the early stage of Maillard reaction.

3.4.2.5 Effect of enzymes on solubility of SoyComil K

Contamination of SoyComil K by insoluble carbohydrates (cellulose hemicellulose, pectin, and trace amount of starch) could have resulted in a protective layer around the soy protein during industrial preparation, which could be reason for its resistance to heat treatment. Therefore, it was treated by carbohydrate hydrolysis enzymes (α-amylase, cellulase, hemicellulase, pectinase), and proteinase K before heat treatment. Enzymatic hydrolysis can be responsible for (1) a decrease in hydrolysate molecular weight (2) increase in ionisable group number, and (3) exposure of previously concealed hydrophobic groups (Lamsal *et al*., 2007). The results show that:

Treatment of SoyComil K by α-amylase prior to heat treatment increased its solubility more than heat treatment only at the same conditions of pH and concentration up to 80°C (Figures 3.12 and 3.13). To my knowledge, no work has been reported to date evaluating the effect of α-amylase on solubility of soy proteins. Oligosaccharide (e.g sucrose and lactose) and polysaccharides (e.g starch and pectin) contents in soy protein concentrate is approximately 11.1% (Abdel-Aziz *et al*., 1997), which may contribute to decreased solubility of SoyComil K by forming a protective layer around the soy protein causing a decreased susceptibility to heat treatment. α-amylase might gradually remove the protective layer of polysaccharides at the surface of protein rendering it more susceptible to heat treatment. The time of both heating and mixing of SoyComil K (pH9) with α-amylase were

increased with the aim to increase solubility even more. Results show a gradual increase in solubility with incubation time, up 80°C to 24 hours compared to the control (Figure 3.13). However, increasing heating temperature to 100°C after treatment by α-amylase for different periods showed no increase in solubility, probably because at 100°C both soy protein units (glycinin and β-conglycinin) are denatured (Renkema and Van Vliet, 2002). At this temperature (100°C) the interior of the tertiary structure of soy protein is disrupted (Elizalde et al., 1996), which results in exposure of hydrophobic regions originally buried within the protein molecule to the aqueous phase, leading to decreased solubility. Soycomil in Figures 3.9 and 3.10 was heated at 100 °C for 10min and showed increased solubility. On the other hand, heat treatment 100 °C for 1h caused a decrease in solubility (Figurs 3.13 and 3.14). The solubility decreased as the heating time was increased, indicating that the hydrophobic characteristic of protein surface was enhanced as heating time was increased (Zhu and Damodaran, 1994). On the other hand, enzymatic hydrolysis (α-amylase), might have gradually remove the protective layer (starch) at the surface of protein rendering it more susceptible to heating time. Other enzymes were used (cellulase and hemicellulase) to attempt to hydrolyse the insoluble carbohydrates (cellulose and hemicellulose) present in SoyComil K, resulting in no significant increase in solubility. The sample treated by pectinase decreased in solubility (1.29%) compared to control (8.42) as shown on Figure 3.12. This might be because the pH of the sample had to be adjusted to pH 4.5, which is the optimum pH of pectinase activity ((manufacturer's instructions of Sigma campany in UK).

Heating of SoyComil K (6%, pH9) to 80°C after treatment by proteinase K was carried out to attempt to increase solubility even more. Sample solubility increased from 15.5% to 20.8% (Figure 3.14). This could be due to the reduction of its secondary structure due to cleavage into smaller polypeptide units by the protease (Kong *et al.,* 2008). However, heat treatment of sample to 100°C after hydrolysis by proteinase K, resulted in decreased solubility (Figure 3.15), which might have arisen from increase in exposed hydrophobic residues leading to increased hydrophobic interaction between peptides (Lamsal et al., 2007).

Overall the treatment of SoyComil K by proteinase K and carbohydrate hydrolysis enzymes increased the solubility more than heat treatment alone at the same pH and concentrations up to 80°C. However, treatment at 100°C for 1h decreased the solubility, due to increased hydrophobic interactions.

3.4.2.6 Intramolecular bonds in Soycomil K dispersions

3.4.2.6.1 Effect of solubilisation in different buffers on particle size of Soycomil K

The stabilization of the soy protein structure may involve electrostatic bonding, hydrophobic bonding, and disulfide linkage (Utsumi and Kinsella, 1985). In this study, we have attempted to identify only the largest forces that lead to the structural changes of SoyComil K caused by heat treatment. Dispersing non-heated and heated (80°C/10 min) SoyComil K in solvent containing reagents (0.3M NaCl, 0.2M 2-mercaptoethanol, and 8M urea), can disrupt selective interactions among polypeptides and subunits, which could lead to reduction in particle size that should reflect the molecular forces contributing to maintenance of protein structure (Zhong *et al*., 2006). Hydrophobic interactions and hydrogen bonding can be disrupted by urea (Zou et al., 1998) whereas 2-mercaptoethanol reduces disulfide bonds to sulfhydryl groups. The electrostatic interaction between proteins or peptides can be disrupted by addition of NaCl, where the counter-ions of NaCl interrupt electrostatic interactions leading to the breakdown of electrostatic bonds (Damianou and Kiosseoglou, 2006). The results displayed in Fig 3.15 indicate that hydrophobic and disulfide bonds are more important in maintenance of the SoyComil K structure than electrostatic interaction.

3.4.2.6.2 Effect of pH, temperature and protein concentration on SH groups of SoyComil K

Sulfhydryl (SH) groups and disulfide (S-S) bonds influence significantly the functional properties of food proteins and play important roles in the formation of relatively rigid structures such as protein gels or dough (Shimada and Cheftel, 1988, Opstved *et al*., 1984 and Ou *et al*., 2004). Heat-induced change in SH group and S-S bonds result in transformation between SH and S-S group, thus the content of SH group and S-S bonds and their change are often assayed when exploring the properties of protein in foods (Shimada and Cheftel, 1988 and Ou *et al*., 2004). In this study, it was observed that as the pH increased the total SH group increased, because S-S bonds are degraded by alkali treatment, which involves an SH group intermediate (Shimada and Cheftel, 1988 and Monahan *et al.*, 1995). On the other hand the increased pH could have caused increase in solubility (Section 3.3.6.2), resulting in more accessibility of SH-groups to DTNB reagent. SoyComil K has the lowest SH groups at pH4.6 (Figure 3.16), which is close to the isoelectric point of soy protein, where the electrostatic repulsive charge is at it lowest and proteins aggregate

resulting in reduced exposure of SH to DTNB reagent (Malhotra and Coupland, 2004). SH-groups increased as temperature was increased. Heat treatment causes exposure of hidden SH groups bonds as protein unfolded, reflecting an increased denaturation degree (Wong and Kitts, 2003). The compact globular structure of soy protein have the hydrophobic regions in the interior, which are stabilized by disulfide bonds (Lamsal et al., 2007) and the protein can not be fully denatured until the interior tertiary structure is disrupted. Therefore, it requires more intensive thermal treatment to be fully denatured. The SH groups decreased markedly as protein concentration increased since the oxidation of SH group to S-S bonds is favored at high protein concentration due to increased protein-protein interaction (Shimada and Cheftel, 1988 and Sorgentini et al., 1995). It can be concluded from the data presented in this study that the SH group increased with increased temperature and pH and decreased with increased protein concentration and decreased pH.

3.4.2.7 Relationship between solubility and hydrophobicity in SoyComil K

Hydrophobicity is the association of non-polar groups or molecules in an aqueous environment, which arises from the tendency of water to exclude non-polar molecules (Kaliszan, 1998). Hydrophobicity of protein is an important structural property since it makes possible to predict its behaviour at oil/water interfaces (Petruccelli and Añón, 1994) and is an important factor that controls protein solubility (Nakai, 1983). Measurement of hydrophobicity might be useful to detect subtle changes in protein structure and functionality that occurs during processing. The highest and lowest solubility of SoyComil K was chosen at pH (4.6, 6.5 and 9), at concentrations of 6% and 18%, and at temperatures (RT, 50, and 80°C) to show the relationship between hydrophobicity and solubility. Hydrophobicity data in Table 3.1 indicate that hydrophobicity increased as solubility increased at all pH and temperatures. Decreased hydrophobicity correlated with decreased solubility, probably due to formation of protein aggregates which buried the hydrophobic sites (Jung *et al*., 2005). Decreased hydrophobicity correlating to decreased solubility can be explained in two ways (a) at low solubility more hydrophobic proteins undergo aggregation, so that only the hydrophilic ones remain soluble; and (b) as the proteins aggregate, hydrophobic zones are hidden, leaving soluble aggregates with low surface hydrophobicity (Wagner *et al.*, 2000).

3.4.3 Properties of SoyComil K emulsions

3.4.3.1 Effect of different treatments on oil droplet size of SoyComil K emulsions

Soy proteins aid in the formation of emulsion by decreasing the interfacial tension between water and oil. However, soy proteins, because of their globular aggregated structure, do not unfold and adsorb at the interface, but rather form a thick interfacial layer, which acts as a physical barrier to coalescence (Roesch and corredig, 2003). The average droplet size distribution (D3,2, surface weighted mean) of SoyComil K emulsions from heated and non heated suspensions was measured by laser light diffraction using a Mastersizer 2000 instrument. Results suggest that heating before homogenization improved the emulsification properties of SoyComil K (Figure 3.17), probably due to dissociation of aggregates and/or unfolding of globular proteins (Roesch and Corredig, 2003). Emulsions of nonheated SoyComil K behaved poorly as emulsifiers, which is shown by the high droplet size corresponding to surface weighted mean (D3,2)**.** High D(3,2) was observed for non-heated SoyComil K due to lower surface activity or due to the fact that the interfacial film formed was less resistant to coalescence during homogenization (Comas *et al*., 2006). There was no considerable size difference in oil droplets of emulsions formed by soyComil K hydrolyzed by enzymes in same heat treatment (Figure 3.17).

3.4.3.2 Effect of different treatments on emulsion stability of SoyComil K

Emulsion stability caused by proteins can be used to define how these proteins can be added to existing foods and how they can replace more expensive proteins traditionally used. The stabilizing effect of proteins in emulsions results from the protective barrier they form around fat droplets, which further prevents their coalescence (Kinsella, 1979). Long –term stability of emulsions depends basically on the thickness and strength of adsorbed protein films at the oil- water interface (Zayas and Lin, 1989). SoyComil K is a poor emulsifier, which is expressed in the large droplet size and phase separation for non-treated SoyComil K (unheated control) corresponding to D(3,2) (Figures 3.17 and 3.18). Chemical, physical and enzymatic modifications have been applied to SoyComil K to improve emulsifying properties. Stability of emulsions made with non-heated SoyComil K at 25°C at different treatments showed separation on the first day and an increase in droplet size (Figures 3.17, 3.18). Stability of emulsions of heated SoyComil K was improved, because thermally treated soy protein prevented creaming and coalescence of emulsions (Comas et al., 2006). This would suggest that either heat treatment made the protein more

surface active or more of the protein became associated with the interface as part of adsorbed protein aggregates.

3.4.3.3 Rheological measurements

3.4.3.3.1 Effect of different treatments of SoyComil K on emulsion viscosity

Viscosity of oil-in-water emulsions is a measure of the emulsion resistance to flow (Raikos, 2006 and Añón, et al., 2001). Viscosity can be used to evaluate the thickening ability of soy proteins that are of practical interest in fluid foods. The viscosity of protein dispersions is mostly influenced by the hydrodynamic properties (molecular weight, size, hydration, frictional ratio and shape of the molecule) of the component protein (Zheng *et al*., 2008). Emulsions prepared with SoyComil K, which had undergone different treatments (heat treatment, enzymatic hydrolysis "proteinase and α-amylase", and mixed with 10%glucose) had studied (Figures 3.23, 3.24, 3.25 and 3.26). Emulsions of heated SoyComil K solutions had under all circumstances a higher viscosity than non-heated emulsions. Viscosity increased as a result of water uptake by the system following hydrogen bond disruption due to heating (Koksel *et al*., 2008). Increase in viscosity may be due to different factors such as a variation in viscosity of the aqueous phase itself, a change in the volume fraction of solute, an increasing droplet concentration and/or variation in the droplet size (Granger *et al.*, 2003). Increased viscosity could be due to decreased oil droplet size of emulsions (Figure 3.17) which increased the total surface area of the emulsions (Granger *et al*., 2005). The results show viscosity decreased as the shear rate increase, showing non-Newtonian rheological behaviour, because at high shear rates the polymer network of emulsion completely breaks up due to shear forces, (Herrera *et al,* 2008).

3.4.3.4 Confocal laser scanning microscopy of SoyComil K emulsions

Confocal laser scanning microscopy (CLSM) minimizes scattered light from out-of-focus structures, and permits the identification of several compounds through use of different fluorescence labels. Therefore, CLSM can be applied as non-destructive visualization technique for micro-particles. Moreover, CLSM allows visualization and characterization of structures not only on the surface, but also inside the particles, provided the material is sufficiently transparent and can be fluorescently labelled. By collecting several coplanar cross sections, a three-dimensional reconstruction of the inspected objects is possible (Lamprecht *et al*., 2000). In the current study, images of emulsions of SoyComil

K treated at different conditions (heat treatment, enzymes “proteinase K and α-amylase” and 10 % glucose) have been produced in order to identify any micro-structural difference. Therefore, as shown in Figures 3.27, 3.28, 3.29, 3.30, 3.31, 3.32, 3.33 and 3.34 the droplets size of emulsions from non-heated SoyComil K solution looks larger than of emulsions from heated protein solutions. This is also a confirmation of average droplet size (D3,2) of non-heated emulsions being larger than heated emulsion (30.05μm, versus 7 μm (Figure 3.17). The much larger apparent droplet size in confocal images as compared with these measured by Master Sizer 2000, could be a result of emulsion instability.

3.5 Conclusions

I- The highest increase in solubility was obtained by heat treatment of 6% SoyComil K, pH9 at 100 °C for 10 min, the solubility increased from 8.42% to 30%, while at 80°C for 10 min was obtained with 0.1M NaCl (6% SoyComil K) at pH 9, the solubility increased from 8.42% to 9.6%.

II- Heat treatment in the presence of sugars

The highest increase in solubility was obtained with heat treatment of 6% protein pH 6.5 with sucrose (1%), at 70°C for30 min. The solubility increased from 2.44% to 7%. The highest degree of glycation was obtain with glucose and lowest was with sucrose. Under these heating conditions (70°C for 30 min), glycation increased the solubility of Soycomil (chapter 3 sections 3.3.2.4 and 3.3.2.4.1).

At high heating temperature (100°C), glycation caused a decrease in solubility probably due to increased covalent protein cross-linking via Maillaird reaction.

III- Enzymes treatment

The highest increase in solubility (12.82%) was obtained with α-amylase after heat treatment (6% soyComil K pH 9) at 80°C for 10 min, this could have been due to removal of a protective layer of insoluble soy polysaccharides which co-purified with the protein during the manufacturing process (chapter 1 section 1.5.2) rendering it more susceptible to heat treatment. However mixing with α-amylase for 24 hours followed by heat treatment at 100°C decreased the solubility (from 30% to 24 % compared to control), due to increased hydrophobic interactions. Treatment by proteinase K increased solubility (20.8%) of SoyComil K (6% at pH9) at 80 °C for 1hour. This could be due to the enzymatic release of smaller polypeptide units. However, treatment by proteinase K followed heat treatment at 100°C decreased the solubility (from 30% to 20.4% compared to control), due to increased hydrophobic interactions.

IV- SoyComil K emulsions

SoyComil K treated with glucose resulted in emulsions with the smallest average droplet size (section 3.3.3), indicating improved emulsifying ability. Since Soycomil treated with glucose had the highest glycation degree, it can be concluded that increased glycation with glucose correlated with increased emulsifying ability and water holding capacity.

V- Rheological measurements (emulsion viscosity)

Emulsions of heated SoyComil K solution (SoyComil K emulsions as prepared in section 2.2.3.1) had under all circumstances a higher viscosity than non-heated emulsions.

Over all in the present study, attempts were made to increase solubility of Soycomil by physical, chemical and enzymatic modifications, and also to improve functional properties such as emulsification. This study has been undertaken to investigate ways to improve its solubility, as well as understand the molecular basis of its insolubility to apply at industry applications. The results show that SoyComil K used in this study had a denaturation degree 27.99% compared to native laboratory prepared soy protein concentrate and hydrophobic and disulfide bonds are the main bonds maintaining the SoyComil K structure. Solubility of SoyComil K was low compared to commercial soy protein concentrates (Arcon ® SJ) where their highest solubility at pH9 and heat treatment (80°C for10 min) are 8.42 (SoyComil K) and 28.5% (Arcon ® SJ. This is confirmed by gel electropherisis, where Soycomil K has lower intensity and number of bands than Arcon ® SJ and SPI at the same concentration and pH under non-reducing conditions. However the band intensity looks similar under reducing condition due to breakdown of disulfide bonds, which is responsible of SoyComil K insolubility. This is also confirmed by total SH groups-content of nonheated SoyComil K 8% (pH7) which was 0.385 μmol/gm compared to that of SPI 8% (pH7), which was 14.8 μmol/gm (Campbell *et al.*, 2008). This provides evidence that SoyComil K has more S-S bonds than SPI which could be responsible for its insolubility.

The processing methods for laboratory preparation of native soy protein concentrates (SPC) and SoyComil K is illustrated in chapter 2 and chapter 1 (chapter 2 section 2.2.1 and chapter 1 section 1.5.2). The main difference between the processing of native soy protein concentrates (SPC) and SoyComil K is the use alcohol (ethanol). Using ethanol during processing method of SoyComil K reduced its solubility, due to denaturation of protein by aqueous alcohol (Zheng *et al.*, 2008), which resulted in protein aggregation due to the removal of water surrounding the protein molecules (Mccurdy, 1990; Hua et al., 1996). Van der Aar et al., (1982) suggested that the combination of alcohol and water in the solvent changed the three dimensional structure of the protein, resulting in fewer proteins soluble in aqueous solutions after treatment.

Insolubility of SoyComil K could also be due to its reaction with insoluble fibers during its processing which could stabilise its tertiary structure and make it more insoluble. Treatment by carbohydrate hydrolysis enzymes followed by heat treatment increased solubility, because enzymes might remove the protective layer of polysaccharides at the surface of protein rendering it more susceptible to heat treatment. Treatment by proteinase K increased solubility of SoyComil K. This could be due to the reduction of its secondary structure and to the enzymatic release of smaller polypeptide units.

Glycation of SoyComil K was caused by all sugars and increased solubility at low temperature (70ºC). No development of yellow or brown colour was visually noticeable in any of the sample. It is concluded that these results reflect glycation in the early stage of Maillard reaction. Under different treatments (enzymes, sugars, salt, heat treatment, and different pH) we increased the solubility from 0.781% to 30% and we have also succeeded to improve emulsification properties.

The following novel findings in this study:

1) The insolubility of Soycomil was due to increased disulfide bond content

2) The solubility of Soycomil K was increased by heat treatment at certain protein concentration and pH and by amylase digestion

3) Emulsifying ability of SoyComil K was improved as shown by increased viscosity and water binding of emulsions

Chapter four

Soy Protein Isolate and Polysaccharides in model yogurts

4.1 Introduction

Food is complex systems composed by different components, in which proteins and polysaccharides play an important role due to their functional and textural properties (Molina Ortiz et *al*., 2004). With the increasing cost of traditional protein sources, more food manufactures are investigating and utilizing soy proteins in dairy type products. Soy protein isolates added as a replacement for the non-fat dry milk in the production of yogurt increased the viscosity and gel strength (Kolar *et al,* 1979). Yogurt products have achieved considerable economic importance worldwide owing to their high nutritional image (Guggisberg et al., 2007). The average composition of yogurt is 4-6% protein, 7-10% carbohydrate and fat 1-3% and rest moisture (Early, 1998; 1980; Yazici and Akyun, 2004). Traditionally, yogurt is a food produced by culturing milk or reduced fat milk using a mixed culture of *lactobacillus bulgaricus* and *streptococcus thermophilus*. The milk is incubated with culture at 43°C until the pH reaches 4.5, then the yogurt is stored at 5 ± °C; however, the process can vary from country to country (Guggisberg et al., 2007).

Polysaccharides play important roles as thickening, stabilizing and gelling agents in many foods (Nakamura *et al*., 2006). Addition of polysaccharides could increase the water binding capacity of soy proteins/polysaccharide dispersions, emulsions and gels and improve textural quality (Hua *et al*., 2003 and Lindhorst, 2000). The stabilization of soy gel structures may involve electrostatic bonding, hydrophobic bonding and disulfide linkage (Utsumi and Kinsella, 1985 and Dickinson and Hong, 1995). Addition of polysaccharide could increase the water binding capacity of soy proteins and improve textural quality (Hua et al., 2003). The most important property of polysaccharide polymers is their high viscosity in solution in a wide range of pH and temperature. Thus it can be used mainly in the food industry as a thickener, stabilizer and dispersion agent (Aydinli and Tutes, 2000).

Glycation of protein is also an effective method for improving the functional properties of protein (Wooster and Augustin, 2007, Saeki, 1997). Maillard-type protein-polysaccharide conjugates have been proposed to be useful as safe functional biopolymers, since the conjugates can be prepared by binding the free amino groups of proteins to the reducing-end carbonyl groups of polysaccharides. Most of the reported reactions however, have been carried out in the dry state of the protein powder and very little scientific literature is available on glycation in the liquid state, resulting in improved functionality. Furthermore, very little is known about functionality of globular proteins, denatured and glycated simultaneously.

4.2 Materials and Methods

In this study yoghurts were manufactures with SPI and polysaccharides as described in Chapter 2 section 2.2.4.

The yoghurts consisted essentially of acidified SPI emulsions.

The sequence of the present study was as follows:

1. SPI dispersions were heat treated in the absence and presence of glucose and/or different polysaccharides (Chapter 2 section 2.2.3.3)

a. The inter- and intra molecular bonds in these dispersions were studied (Chapter 2 sections 2.2.6.2 and 2.2.6.3)

2. The SPI dispersions of (a) were then emulsified with oil with the aid of homogenisation (Chapter 2 section 2.2.3).

   b) The effects of the following parameters on emulsifying properties SPI (oil droplet size of emulsions) at pH 7 were determined (Chapter 2 section 2.2.3.2 and 2.2.8).

- SPI heated with glucose
- SPI heated with different polysaccharides (pectin, amylopectin, starch, xanthan, carrageenan, guar gum and locustbean gum)
- SPI heated with glucose and polysaccharides (The effect of glucose was studied to simulate a yoghurt emulsion where glucose would serve as the carbon source for yoghurt cultures)
- Homogenisation (described in Chapter 2 sections 2.2.3.2 and 2.2.8.1.2)

3. The emulsions were then acidified and the following properties of the resulting yoghurts were measured (Chapter 2 section 2.2.3.3):

- Gel Hardness
- Water binding capacity
- Types of intermolecular bonds

4.3 Results

4.3.1 Intra-molecular bonds in a commercial soy protein isolate dispersion

As mentioned in section 3.2.2.6.1 molecular interaction studies were performed with 6% SoyComil K at pH9. Identical conditions were used to identify the intermolecular bonds of SPI dispersion. Intra-molecular forces operate within the molecular or fundamental units of a substance (ionic interaction, hydrophobic interaction, hydrogen bonds, covalent bond, and Van der Waal forces) (Chapter 1 section 1.6). Dispersing of commercial soy protein isolate (6%) pH9 (treated at room temperature and 80°C for 10 min) in imidizole solvents containing one of the reagents (0.3M NaCl, 0.2M 2-Mercaptoethanol, and 8M urea), could disrupt selective interactions among polypeptides, which could lead to reduction in particle size. The effect of each of the three reagents on particle size of protein should reflect the molecular force contributing to maintenance of protein structure (Zhong et al., 2006). For the non-heated sample, the smallest particle size was obtained by treatment with NaCl (26μm) indicating non-heated SPI mainly maintained by electrostatic bonds. The next was found with disulfide bond breaking reagent (0.2M 2-mercaptoethanol, and then 8M urea, (Figure 4.1). For the heated SPI samples, treatment with urea and mercaptoethanol resulted in the smallest particle size (19.626 μm and 19.836 μm respectively) indicating heated SPI mainly maintained by hydrophobic and disulfide bonds.

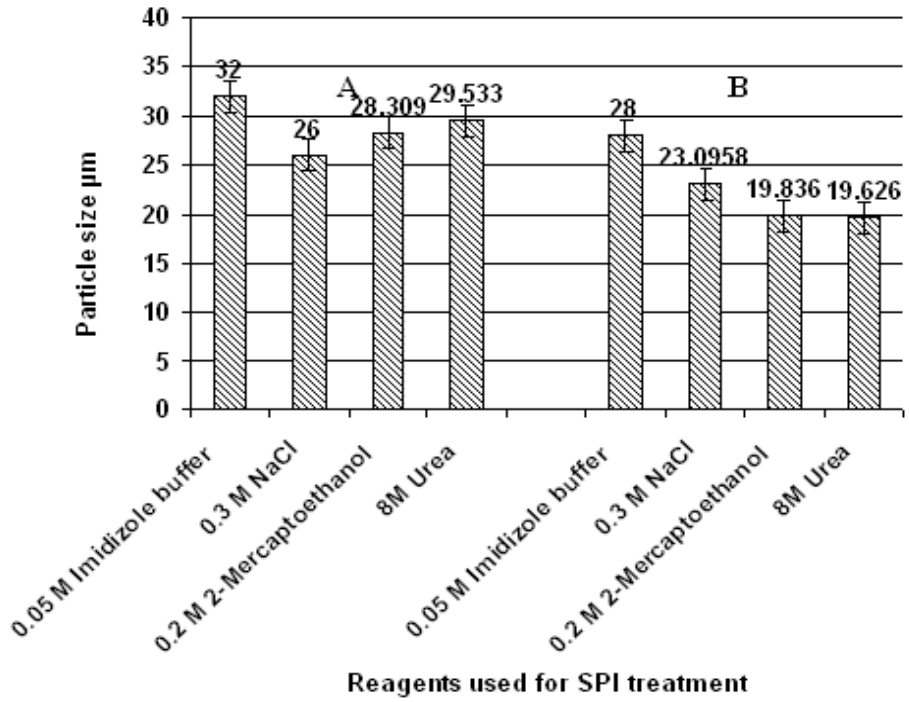

Figure 4.1 Particle size profile of 6% soy protein isolate pH9 dispersion treated with different reagents

Where A: Treatment at room temperature (22± 3°C) and B: treatment at 80°C for10min

4.3.2 Glycation degree of commercial soy protein isolates

Glycation degree was measured in suspensions of commercial SPI heat-treated in the presence of glucose and/or different types of polysaccharides (11% glucose, 0.5% starch, 0.1% locust bean, 0.1% carrageenan, 0.1% guar gum, 0.1% xanthan, 0.5% amylopectin, and 0.5% pectin). The effect on reduction of free amino groups concentration is presented in Figure 4.2. The free amino groups of SPI was expressed in terms of L-leucine. Decrease in available aminogroups compared to control indicating that glycation occurred with sugars. The result show the highest available amino groups (1.71µg/µl) was obtained with SPI heated without glucose or polysaccharides, whereas the lowest available amino groups were obtained by pectin and amylopectin (1.62 and 1.63 µg/µl respectively)., were the values for pectin and amylopectin significantly less than glucose at Confidence

levels were set at 95% (n=3, $p<0.05$). The lowest glycation degree was obtained with starch, which has highest available amino groups (1.7 µg/µl) compared to other sugars (Figure 4.2)

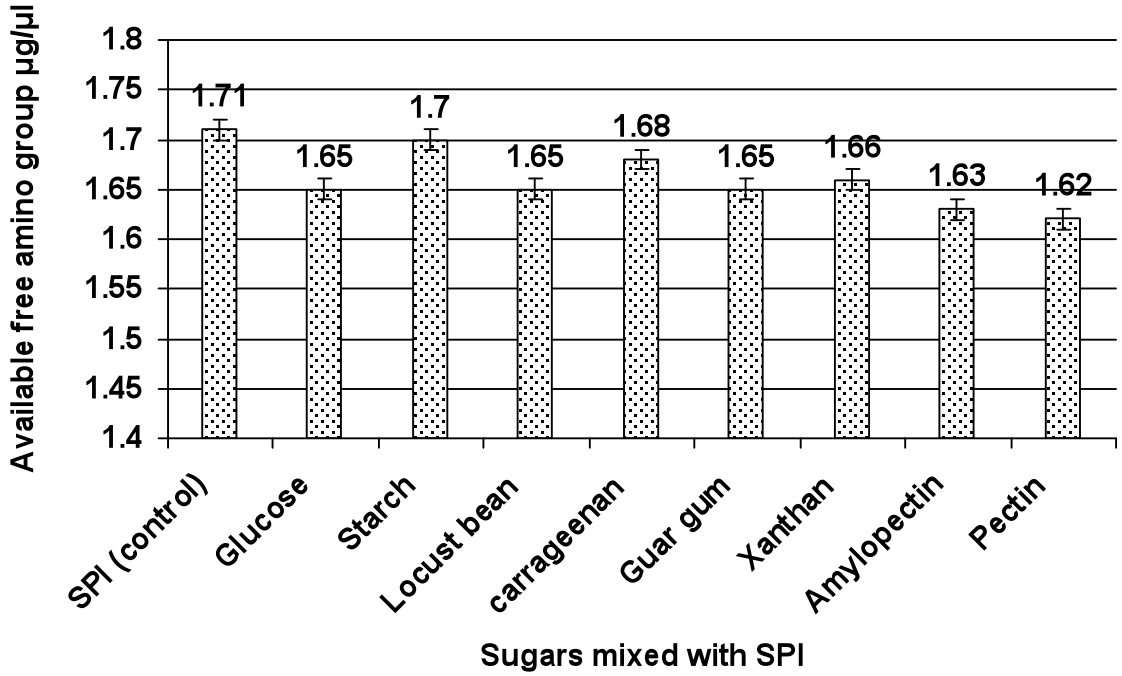

Figure 4.2 Available amino groups of SPI heated (95°C for 30min) in the presence of glucose or polysaccharides

In present study glycation of SPI was investigated by measuring the available free amino groups. Glycation is defined as the decrease in available amino groups of SPI when incubated for a certain time and temperature in presence of reducing sugars (chapter 1 section 1.7.1.5). In the case of starch, which has no reducing groups (Oste *et al.*, 1990), heat treatment resulted in no glycation. Pectin and amylopectin showed highest glycation (lowest available amino groups). Amylopectin has more than one reducing group in its structure, while pectin has an anionic nature, which enhances covalent linkage and was shown to play an important role in the Maillard reaction (Al-Hakkak and Kavale, 2002). It has been shown that polysaccharides with reducing properties may enhance functional properties of protein to a higher extent than monosaccharides and oligosaccharides, because

their larger size and net charge lead to important structure changes (Jiménez-Castaño *et al*., 2005). Polysaccharides may have fewer reducing ends per molecular weight, and so have less chance to glycate, but the high molecular weight and branching of polysaccharides may provide the opportunity to increase conjugation to available amino acids. No development of yellow or brown colour was visually noticeable in any of the sample, therefore it is concluded that these results reflect glycation in the early stage of Maillard reaction.

4.3.3 Properties of SPI emulsions

In this, section the effect of following factors on oil droplet distribution of SPI emulsions were studied:

- SPI heated with glucose
- SPI heated with polysaccharides
- SPI heated with glucose and polysaccharides (the effect of glucose was studied to simulate a yoghurt emulsion where glucose would serve as the carbon source for yoghurt cultures)
- Homogenisation

4.3.3.1 Effect of heat treatment of SPI on oil droplets size of 3% oil emulsions at pH 7

Changes in the average droplets size distribution (D3,2 "surface weighted mean") of emulsions at pH 7 made with heated or non-heated SPI are shown in Figure 4.3. Surface weighted mean D3,2 is defined as the diameter of a sphere that has the same volume/surface area ratio as a particle of interest (Liu et al., 2007; Pacek et al., 1998; Downs and Sarv, 2003). The mean droplet size distribution (D3, 2) of the emulsion produced by the heated SPI (45.5μm) was smaller than of the non-heated SPI suspension (48.7 μm) (significant difference at confidence level of 95% $p<0.05$).

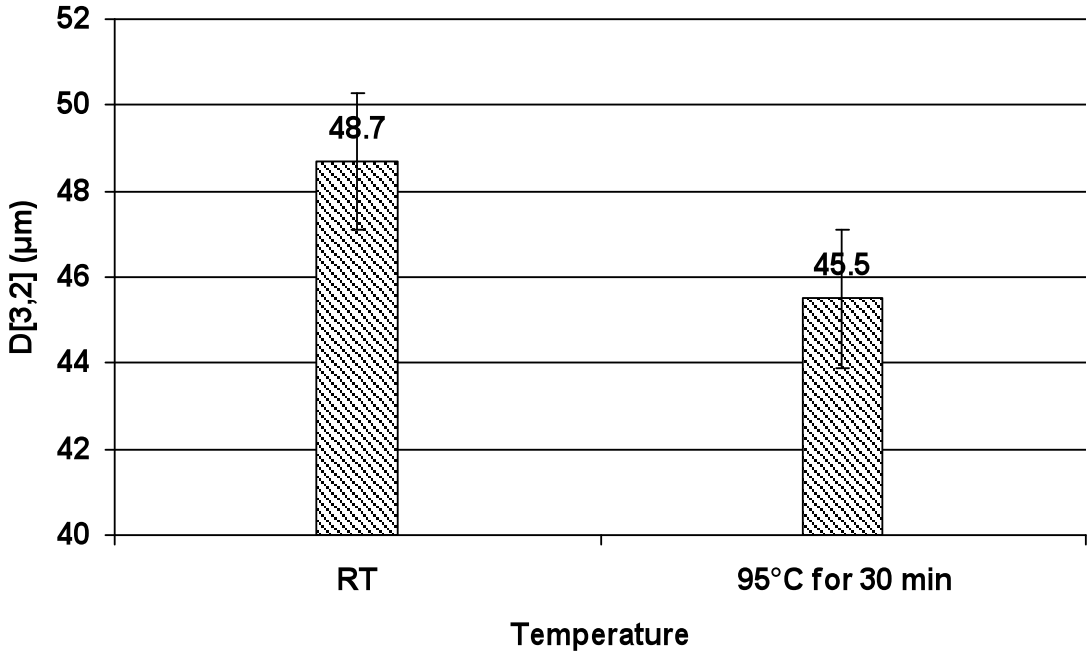

Figure 4.3 Effect of heat treatment of SPI on oil droplets size of 3% oil emulsion pH 7

Where RT: Room temperature (22± 3 °C)

4.3.3.2 Effect of ultra high-pressure homogenization on oil droplets size of SPI emulsions

In this study droplet size (D3,2) of emulsions of heated and non-heated SPI (3% sunflower oil) was studied. Before high-pressure homogenization, emulsion presented large droplet size, whereas after high-pressure homogenization at 200-600 bar, the droplet sizes were reduced (Figure 4.4). The results show **e**mulsion from non-heated and non-homogenized SPI suspension had largest droplets size (48.7 µm), and emulsion from heated and non-homogenized SPI dispersions had smaller droplets size than non-heated non-homogenized emulsion (45.5 µm), but significantly larger than homogenized emulsions. Emulsion from non-heated and homogenized SPI suspension had smaller droplets size than non-homogenized emulsions, but significantly larger than heated, homogenized emulsions, whilst emulsion from heated and homogenized SPI dispersions had the smallest droplets size (Figure 4.4).

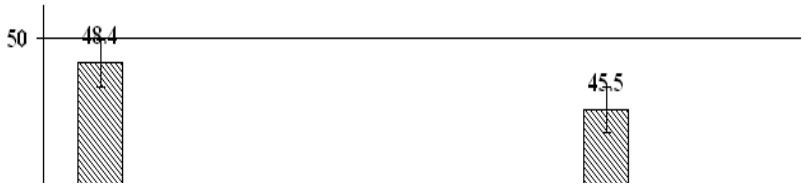

Figure 4.4 Effect of ultra high-pressure homogenization on droplets size distribution of soy protein isolates emulsions

4.3.3.3 Effect of heat treatment of SPI in presence of polysaccharides on oil droplets size of SPI emulsions

Changes in the average droplets size distribution (D3,2;surface weighted mean) of emulsions as function of SPI (heated or non-heated) with different polysaccharides or glucose (11% glucose, 0.5% pectin, 0.5% amylopectin, 0.5% starch, 0.1% carrageenan, 0.1% xanthan, 0.1% guar and 0.1% locust bean) were studied. The average droplet size (D3,2) of all emulsions were measured immediately after emulsion formation. Figure 4.5 difference in droplets size (D3,2) in non-homogenized emulsions contain one sugar (glucose or polysaccharides) and emulsions contain both sugars (glucose and polysaccharides) (un heated and heated to 95 °C for 30 min). The largest oil droplets were obtained with both non-heated and heated SPI and locust bean gum with glucose. The

smallest oil droplets was obtained with both non-heated and heated SPI and amylopectin. In general, heat treatment of SPI in presence of sugars (polysaccharides or glucose) caused increase in droplets size, compared to control (SPI only). Heat treatment of SPI in presence sugars increased droplet size. While decrease droplet size of control (SPI only), were significantly smaller than that of non-heated SPI. Combination of glucose with polysaccharides increased droplets size more than glucose or polysaccharides alone in all conditions.

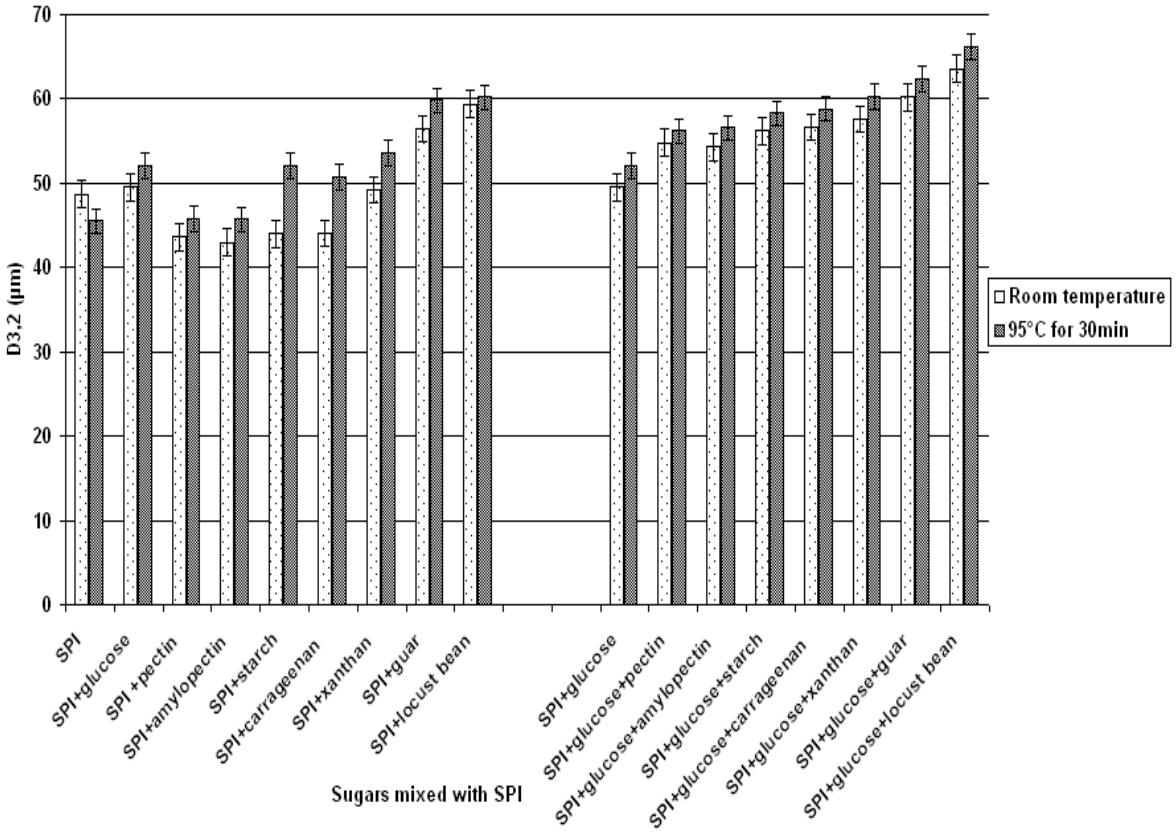

Figure 4.5 Oil droplet size of non-homogenised emulsions made from SPI in the presence of glucose or polysaccharides or glucose combined with polysaccharides

For homogenised emulsions Figure 4.6 show difference in droplets size (D3,2) in emulsion contain one sugar (glucose or polysaccharides) and emulsions contain both sugars (glucose and polysaccharides) at room temperature and 95 °C for30 min. The largest oil droplets were obtained with both non-heated and heated SPI and locust bean gum with glucose. The

smallest oil droplets were obtained with both non-heated and heated control (SPI only). Homogenization decreased droplets size significantly compared to non-homogenized emulsions (Fig. 4.5). Presence of sugars increase the droplets size compared to control for under both non-heated and heat-treated SPI. Heat treatment decrease droplet size of control (SPI only), whilst combination of glucose with polysaccharides increased droplets size more than glucose or polysaccharides alone in all conditions (Figure 4.6).

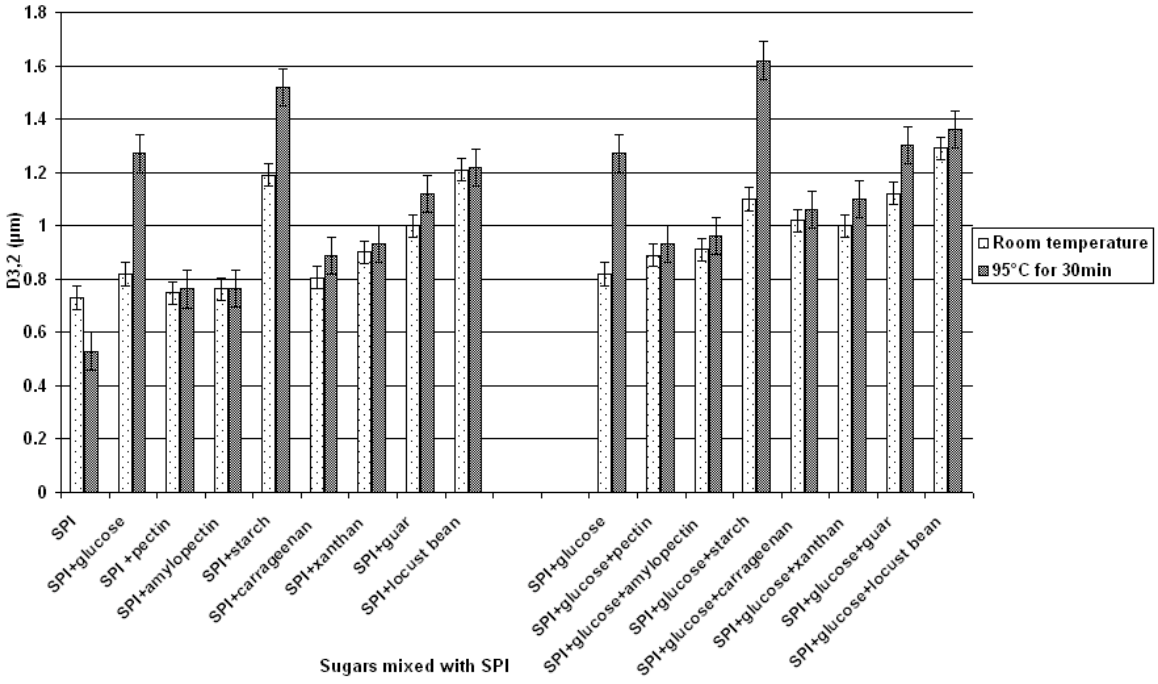

Figure 4.6 Oil droplet size of homogenised emulsions made from SPI heat treated in the presence of glucose or polysaccharides or glucose combined with polysaccharides

4.3.4 Soy yogurts

4.3.4.1 Texture analysis of yogurts

The texture of these laboratory made yogurts (section 2.2.4), as well as commercial soy yogurt (Alpro Soya, bought from Tesco Supermarket UK), was analyzed by measuring three parameters, hardness (Tables 4.1 and 4.2), cohesion strength (Tables 4.1 and 4.3) and adhesiveness (Tables 4.1 and 4.4). Additionally, laboratory yogurts were subjected to heat treatment (95°C for 20 min) to study its effect on texture. The results show highest hardness under all treatments was obtained with yogurt produced from SPI and pectin (0.28- 0.57 N) and lowest hardness under all treatments was obtained with yogurt produced from SPI only (control). In general, yogurts from homogenized emulsions have higher hardness than non-homogenized suspension. Yogurts hardness increased as degree of glycation increased. For all yogurts tested, texture analysis indicated very low cohesion strength and adhesiveness that were below or just above the detection limit of the texture analyzer. No obvious differences in cohesion strength and adhesiveness were observed.

Table 4.1 Textural profile analysis of commercial soy yogurt (Alpro Soya)

Composition	7.6% hulled soya beans (3.8% protein) + 11.3% carbohydrates (sugar 10.7%)+ fat 2.2% + 0.7% fiber (pectin and agar).
pH	4.00
Hardness (N)	0.12
Cohesion strength (N)	_
Adhesiveness (mJ)	_

(—) Value that was below or just above the detection limit of the texture analyzer.

Table 4.2 Hardness (Newton unit "N") of laboratory prepared soy yogurts

Yogurt composition	Non-homogenised, non-heated emulsion (pH 7)		Non-homogenised, heated emulsion pH 7		Homogenised, non-heated emulsion pH 7		Homogenised, heated emulsion pH 7	
	Non-heated yogurt	Heated yoghurt	Non-heated yogurt	Heated yoghurt	Non-heated yogurt	Heated yoghurt	Non-heated yogurt	Heated yoghurt
SPI	0.10	0.18	0.28	0.32	0.32	0.40	0.34	0.42
SPI+ glucose	0.20	0.26	0.33	0.40	0.37	0.44	0.48	0.53
SPI+ pectin	0.11	0.30	0.33	0.38	0.36	0.43	0.38	0.54
SPI+ amylopectin	0.12	0.25	0.34	0.36	0.35	0.47	0.40	0.52
SPI+ starch	0.11	0.22	0.33	0.37	0.35	0.48	0.39	0.52
SPI+ carrageenan	0.11	0.20	0.31	0.43	0.38	0.43	0.40	0.48
SPI+ xanthan	0.13	0.21	0.31	0.39	0.40	0.42	0.45	0.52
SPI+ guar gum	0.12	0.28	0.33	0.38	0.41	0.43	0.47	0.52
SPI+ locust bean	0.13	0.25	0.34	0.40	0.38	0.44	0.41	0.56
SPI+ pectin+ glucose	0.28	0.41	0.44	0.48	0.47	0.52	0.50	0.57
SPI+ amylopectin+ glucose	0.25	0.39	0.42	0.45	0.42	0.49	0.41	0.55
SPI+ starch+ glucose	0.26	0.37	0.40	0.46	0.43	0.48	0.46	0.58
SPI+ carrageenan+ glucose	0.18	0.32	0.35	0.46	0.41	0.46	0.46	0.54
SPI+ xanthan+ glucose	0.18	0.30	0.41	0.46	0.42	0.46	0.45	0.53
SPI+ guar gum+ glucose	0.18	0.31	0.37	0.43	0.42	0.47	0.46	0.51
SPI+ locust bean+ glucose	0.21	0.37	0.40	0.46	0.43	0.50	0.45	0.55

Table 4.3 Cohesion strength (Newton unit "N") of laboratory prepared soy yogurts

Yogurt composition	Non-homogenised, non-heated suspension (pH7)		Non-homogenised, heated suspension (pH7)		Homogenised, non-heated suspension (pH7)		Homogenised, heated suspension (pH7)	
	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt
SPI	-	0.01	0.02	-	-	0.01	0.01	-
SPI+ glucose	-	-	-	-	0.01	-	0.01	-
SPI+ pectin	0.01	0.01	-	-	-	0.01	0.01	0.01
SPI+ amylopectin	-	-	-	-	-	0.01	0.01	-
SPI+ starch	-	-	-	-	0.01	-	0.01	-
SPI+ carrageenan	-	-	-	-	-	-	-	-
SPI+ xanthan	-	0.01	-	-	-	0.01	0.01	-
SPI+ guar gum	-	0.01	0.01	-	-	0.01	0.01	-
SPI+ locust bean	-	-	0.02	0.01	-	0.02	0.01	-
SPI+ pectin+ glucose	-	-	0.01	-	0.01	0.01	0.01	0.01
SPI+ amylopectin+ glucose	-	0.02	0.02	-	-	0.01	-	-
SPI+ starch+ glucose	-	0.01	0.01	-	0.01	0.02	0.01	0.01
SPI+ carrageenan+ glucose	-	0.01	-	-	0.02	-	-	-
SPI+ xanthan+ glucose	-	-	-	-	0.01	-	0.01	-
SPI+ guar gum + glucose	-	-	-	-	0.01	-	0.01	-
SPI+ locust bean+ glucose	-	-	0.02	0.01	-	0.02	0.01	-

Table 4.4 Adhesiveness strength (micro Joules unit "mJ) of laboratory prepared soy yogurts

Yogurt composition	Non-homogenised, non-heated suspension (pH7)		Non-homogenised, heated suspension (pH7)		Homogenised, non-heated suspension (pH7)		Homogenised, heated suspension (pH7)	
	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt
SPI	-	0.01	0.01	-	-	0.01	0.01	-
SPI+ glucose	-	-	-	-	0.01	-	0.01	-
SPI+ pectin	0.02	0.01	-	-	-	0.01	0.02	0.01
SPI+ amylopectin	-	-	-	-	-	0.01	0.01	-
SPI+ starch	-	-	-	-	-	-	0.01	-
SPI+ carrageenan	-	-	-	-	-	-	-	-
SPI+ xanthan	-	0.01	-	-	-	0.01	0.01	-
SPI+ guar gum	-	0.01	0.01	-	-	0.01	0.01	-
SPI+ locust bean	-	-	0.02	0.01	-	0.02	0.01	0.01
SPI+ pectin+ glucose	-	-	0.01	-	0.01	0.01	0.01	0.01
SPI+ amylopectin+ glucose	-	0.01	0.02	-	-	-	-	-
SPI+ starch+ glucose	-	0.01	0.01	-	0.01	0.02	0.02	0.02
SPI+ carrageenan+ glucose	-	0.01	-	-	0.02	-	-	-
SPI+ xanthan+ glucose	-	-	0.01	-	0.01	-	0.01	-
SPI+ guar gum + glucose	-	-	-	-	0.01	-	0.01	-
SPI+ locust bean+ glucose	-	-	0.02	0.01	-	0.02	0.01	0.01

4.3.4.2 Water holding capacity of yogurts

Water holding capacity of laboratory made yogurts (section 2.2.4), was measured, additionally, laboratory yogurts were subjected to heat treatment (95°C for 20 min) to study its effect on water holding capacity (Table 4.5). Results show highest water holding capacity (WHC) under all treatments was obtained with yogurt produced from SPI and pectin with glucose (70%) and lowest WHC was obtained with yogurt produced from SPI only (25%). In general, glucose, polysaccharides, heat treatment and homogenizion increase water holding capacity (Table 4.5).

Table 4.5 water holding capacity (%)of soy yogurts

Yogurt composition	Non-homogenised, non-heated emulsion pH 7		Non-homogenised, heated emulsion pH 7		Homogenised, non-heated emulsion pH 7		Homogenised, heated emulsion pH7	
	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt	Non-heated yogurt	Heated yogurt
SPI	25.0	37.0	35.0	40.0	38.3	42.6	39.7	44.2
SPI+ glucose	31.0	40.2	41.3	43.2	44.5	51.3	47.2	53.0
SPI+ pectin	49.1	53.2	52.0	59.0	55.0	63.0	58.3	65.0
SPI+ amylopectin	38.5	43.5	46.0	50.0	48.3	56.2	51.0	59.0
SPI+ starch	36.0	42.8	45.2	48.1	46.6	55.3	51.0	58.6
SPI+ carrageenan	32.0	41.0	42.2	44.0	45.2	53.8	49.3	54.0
SPI+ xanthan	40.0	45.2	48.0	51.0	49.2	57.0	53.0	60.0
SPI+ guar gum	40.5	44.4	47.0	51.3	49.0	57.9	53.6	59.7
SPI+ locust bean	41.2	44.9	47.7	52.0	50.0	58.6	55.0	60.4
SPI+ pectin+ glucose	55.0	62.0	58.0	66.0	62.2	66.7	67.8	70.0
SPI+ amylopectin+ glucose	43.6	48.4	48.7	53.0	50.1	58.3	53.6	63.0
SPI+ starch+ glucose	40.3	46.0	47.0	52.0	49.0	58.2	53.0	64.0
SPI+ carrageenan+ glucose	40.0	44.6	44.3	50.0	47.2	55.0	52.1	58.3
SPI+ xanthan+ glucose	44.0	49.2	51.0	55.0	51.0	60.1	55.0	66.0
SPI+ guar gum + glucose	44.3	49.1	51.3	56.3	51.6	61.3	56.2	66.4
SPI+ locust bean+ glucose	44.6	50.0	50.9	56.1	52.0	61.0	57.0	67.0

4.3.4.3 Total and free sulfhydryl group contents in yogurts

The amount of free and total sulfhydryl groups in yogurt produced from SPI and pectin (best improved of yogurt and no separation) and yogurt produced from SPI and carrageenan (least improved) were measured. The results in Table 4.6 show that the total SH groups of yogurts ranged between 4.0 to 3.83 μmol/gm, and free SH groups ranged from 3.3 to 3.0 μmol/gm, for both yogurts made from non-heated or heated SPI. There was no significant difference between in total sulfhydryl and free sulfhydryl contents indicating no additional formation of disulfide bonds

Table 4.6 Total and free sulfhydryl contents in soy yogurts

Yogurt composition	Total SH groups (μmol/gm)	Free SH group (μmol/gm)
4.3% SPI (RT)	4.00	3.30
4.3% SPI heated to 95°C/30 min	3.93	3.01
4.3% SPI+ 11%glucose. (RT)	3.85	3.00
4.3% SPI+11%glucose heated to 95°C/30 min.	3.97	3.00
4.3% SPI+0.5% pectin (RT)	3.88	3.04
4.3% SPI+0.5% pectin heated to 95°C/30 min	3.85	3.00
4.3% SPI+0.5% pectin +11%glucose (RT)	3.89	3.12
4.3% SPI+0.5% pectin +11%glucose heated to 95°C/30 min.	3.92	3.20
4.3% SPI+0.1% carrageenan (RT)	3.9.00	3.10
4.3% SPI+ 0.1% carrageenan heated to 95°C/30 min.	3.83	3.10
4.3% SPI+0.1% carrageenan +11%glucose (RT).	3.86	3.20
4.3% SPI+0.1% carrageenan +11%glucose heated to 95°C/30 min.	3.96	3.20

RT: Room temperature (22± 3°C)

4.3.4.4 Total and free sulfhydryl group contents in yogurts

Yogurt produced from SPI and pectin (best improved of yogurt with highest WHC and no separation) and yogurt produced from SPI and carrageenan (least improved with lowest WHC) were dispersed in imidazole solvents containing reagents (0.3M NaCl, 0.2M 2-mercaptoethanol and 8M Urea) to study the effect on solubilization of yogurt. Solubility of gel yogurt in the various solvents should reflect the molecular bonds contributing to maintenance of the gel network structure in yogurts (Utsumi and Kinsella, 1985) (Table 4.7). Results show highest solubility was obtained with yogurts treated by 8M urea in both yogurts produced from non-heated SPI or heat treated SPI, which indicates that the majority of bonds were hydrophobic bonds and lowest solubility was obtained with yogurts treated by 0.3M NaCl or 0.2M 2-Mercaptoethanol. Results indicate that the majority of bonds were hydrophobic bonds, while electrostatic and disulfide bonds were low in maintaining the yogurt structure.

Table 4.7 Solubility profile of soy yogurts in various reagents

Yogurt composition	% Solubility							
	0.05M Imidazole (control)		0.3M NaCl		0.2M 2-Mercaptoethanol		8M Urea	
	RT	95°C/30 min	RT	95°C/30 min	RT	95°C/30 min	RT	95°C/30 min
SPI	0.61	0.72	1.00	1.10	0.75	0.80	10.21	12.52
SPI+ glucose	0.68	0.74	1.00	1.20	0.90	1.00	10.31	12.73
SPI+ pectin	0.70	0.77	1.10	1.40	1.30	1.40	10.43	12.90
SPI+ carrageenan	0.65	0.74	1.00	1.29	1.22	1.42	10.24	12.66
SPI+ pectin+ glucose	0.71	0.83	1.10	1.30	1.46	1.58	10.80	13.00
SPI+ carrageenan + glucose	0.69	0.79	0.95	1.23	1.42	1.49	10.63	12.63

RT: Room temperature (22± 3°C)

4.3.4.5 Confocal laser scanning microscopy (CLSM) of soy yogurts

The microstructure of yogurt produced from SPI and pectin with glucose (highest hardness and WHC) was studied. CSLM pictures (Figures 4.7 A-F) show a clear difference in network structure as a function of polysaccharide and heat treatment. Yogurts produced from SPI and sugars (polysaccharides and glucose) show that the microstructures (droplet size) are fairly homogeneous and more condensed compared to control (yogurt from SPI only).

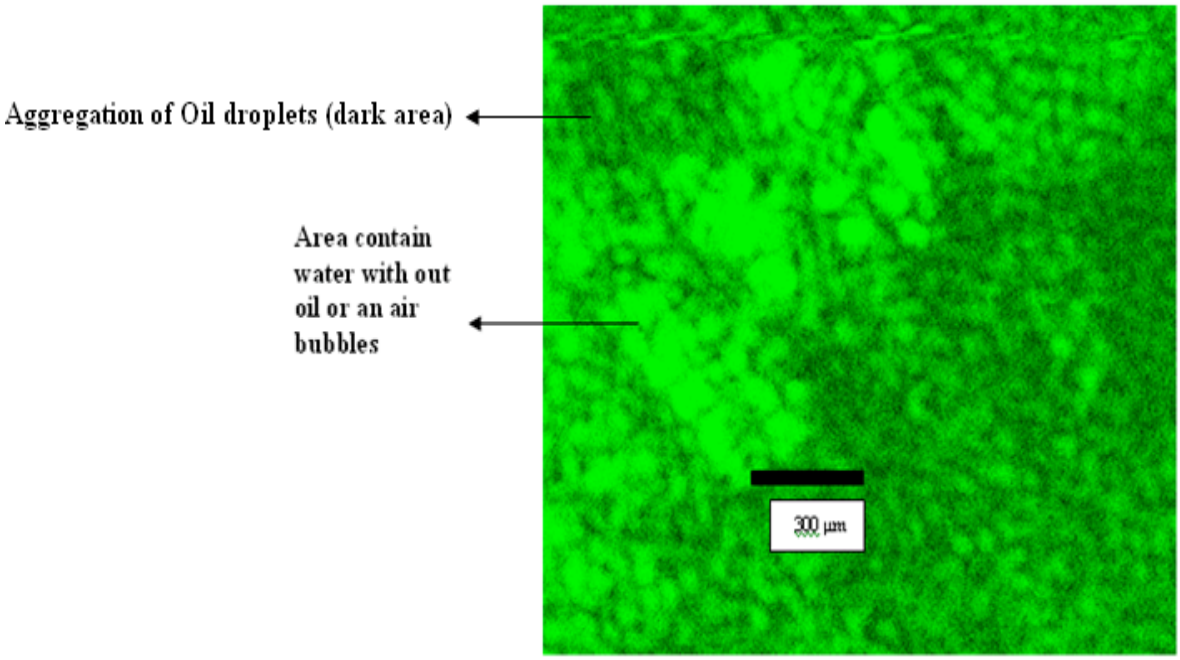

Figure 4. 7 A. Yogurt of heated SPI (control), homogenised.

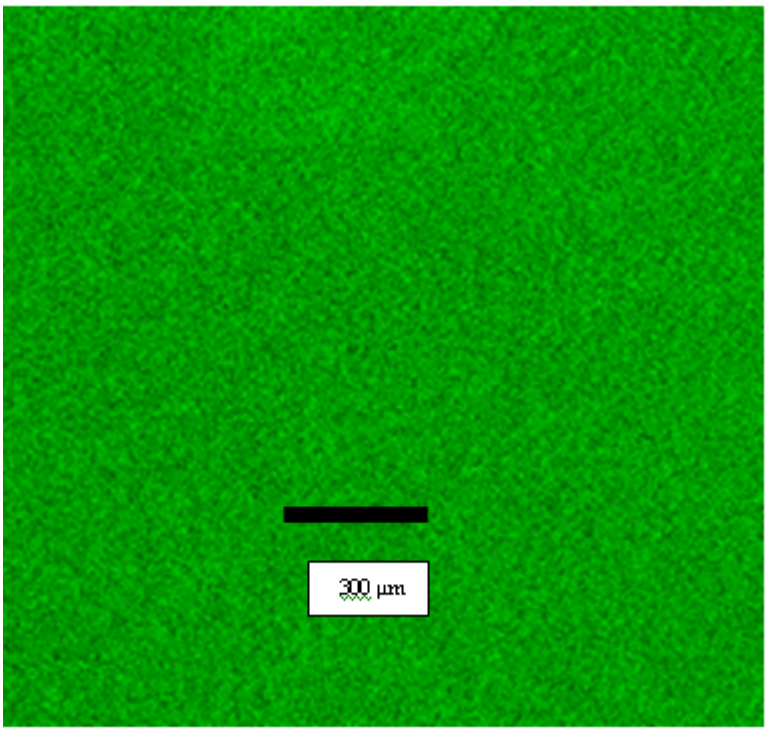

Figure 4. 7 B. Yogurt from heated SPI pH 7, mixed with 0.5% pectin and 11% glucose, emulsified with 3% sunflower oil followed by homogenization and acidification

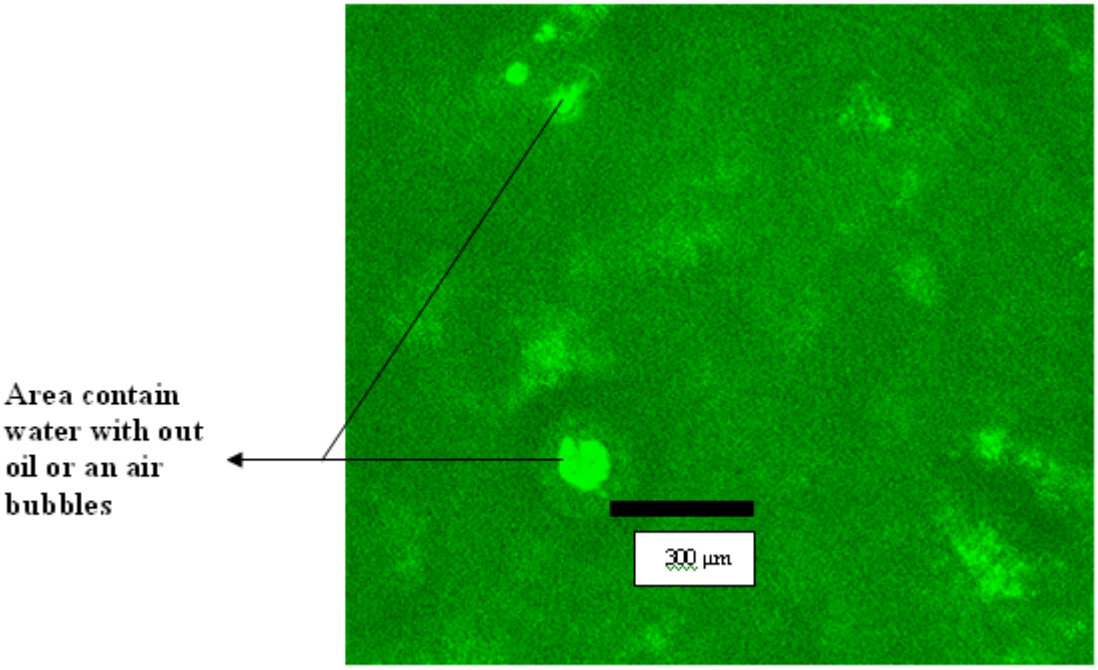

Figure 4.7 C Yogurt from non heated SPI pH 7, mixed with 0.5% pectin and 11% glucose, emulsified with 3% sunflower oil followed by homogenization and acidification

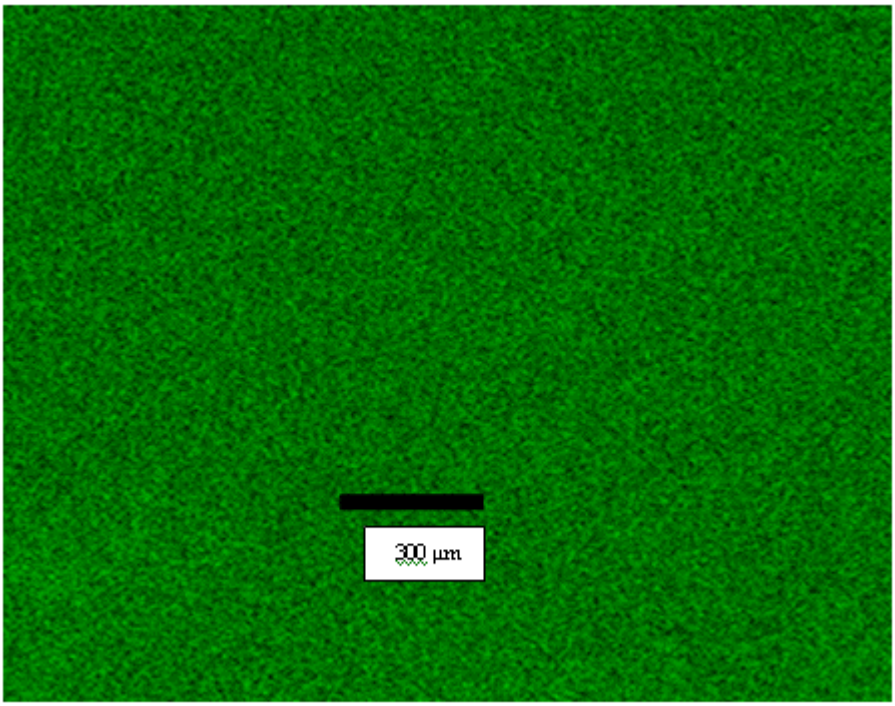

Figure 4.7 D Yogurt of heated SPI pH 7, mixed with 0.5% carrageenan and 11% glucose, emulsified with 3% sunflower oil, homogenization and acidified

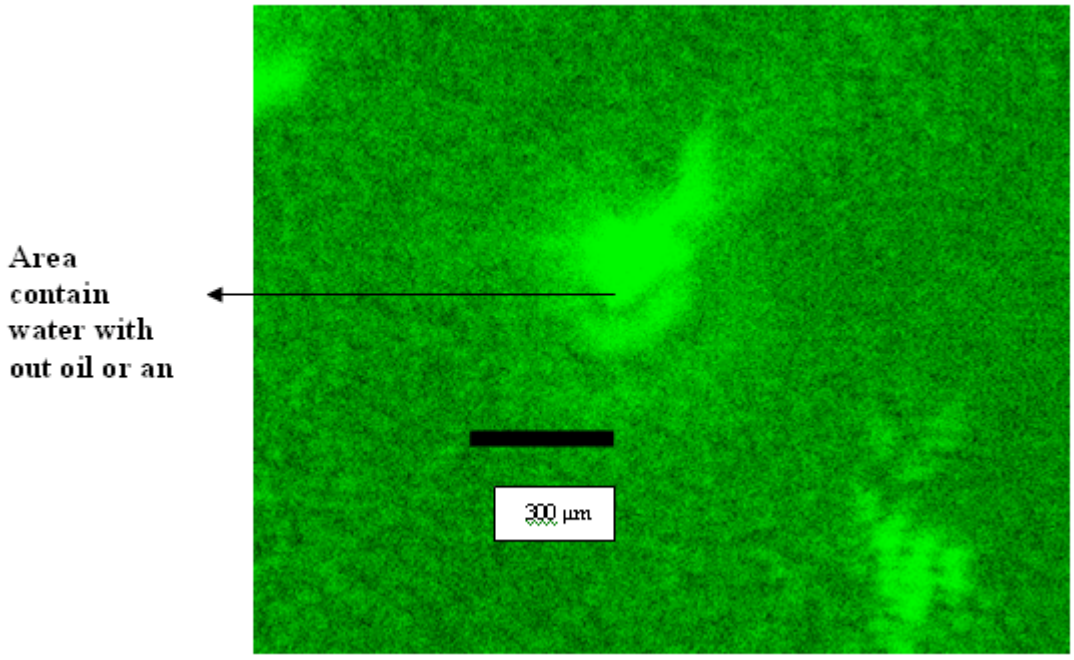

Figure 4.7 E Yogurt of non-heated SPI pH 7, mixed with 0.5% carrageenan and 11% glucose, emulsified with 3% sunflower oil, homogenization and acidified.

4.4 Discussion

4.4.1 Intra-molecular bonds in a commercial soy protein isolate dispersion

Dispersing of commercial soy protein isolate (6%) pH9 (treated at room temperature and 80°C for 10 min) in imidizole solvents containing one of the reagents (0.3M NaCl, 0.2M 2-Mercaptoethanol, and 8M urea), could disrupt selective interactions among polypeptides, which could lead to reduction in particle size. The results show that particle size of heated SPI in all buffers were significantly smaller than non-heated SPI (Fig 4.1). This indicates that the difference between heated and non-heated soy protein is that heat treatment resulted in increased electrostatic bonds, sulfhydryl groups and hydrophobic bonds. Thermal treatment causes dissociation of aggregates (oligomers) in to subunits of smaller molecular weight (Shimada and Cheftel, 1988 and Rangavajhyala et al., 1997) as well as partial denaturation of monomers. A decrease in average particle size was found with the addition of each of the three reagents in both non-heated and heated samples.

For the non-heated sample, the smallest particle size was obtained by treatment with NaCl . The difference between effects of 0.2M 2-Mercaptoethanol and urea was not significant, indicating that disulfide and hydrophobic bonds are low in SPI structure. The results suggest, that non-heated soy protein isolate structure is mainly maintained by electrostatic bonds. For the heat-treated sample, treatment with urea and mercapto-ethanol resulted in the smallest particle size; suggesting that hydrophobic and disulfide bonds are important in maintenance of heated soy protein isolate structure. Puppo and Añón, 1998 showed that hydrophobic interaction is favoured at high temperature treatments.

4.4.2 Glycation degree of commercial soy protein isolates

In present study, glycation of SPI was investigated by measuring the available free amino groups (Figure 4.2). Glycation is defined as the decrease in available amino groups of SPI when incubated for a certain time and temperature in presence of reducing sugars (chapter 1 section 1.7.1.5). In the case of starch, which has no reducing groups (Oste *et al.*, 1990), heat treatment resulted in no glycation. Pectin and amylopectin showed highest glycation (lowest available amino groups; Figure 4.2). Amylopectin has more than one reducing group in its structure, while pectin has an anionic nature, which enhances covalent linkage and was shown to play an important role in the Maillard reaction (Al-Hakkak and Kavale, 2002). It has been shown that polysaccharides with reducing properties may

enhance functional properties of protein to a higher extent than monosaccharides and oligosaccharides, because their larger size and net charge lead to important structure changes (Jiménez-Castaño *et al*., 2005). Polysaccharides may have fewer reducing ends per molecular weight, and so have less chance to glycate, but the high molecular weight and branching of polysaccharides may provide the opportunity to increase conjugation to available amino acids. No development of yellow or brown colour was visually noticeable in any of the sample, therefore it is concluded that these results reflect glycation in the early stage of Maillard reaction.

4.4.3 Properties of SPI emulsions

4.4.3.1 Effect of heat treatment of SPI on oil droplets size of 3% oil emulsions at pH 7

Change of protein functionality due to thermal denaturation may induce changes in protein-stabilized emulsions. The droplet size distribution is an important factor, which strongly influences both the textural stability and sensory characteristics of protein-stabilised oil-in-water emulsions (Kiokias *et al*., 2007). The average droplets size distribution (D3,2) of emulsions made with heated and non-heated SPI are shown in Figure 4.3. The mean droplet size distribution of heated SPI emulsion was smaller than of the non-heated version. Denaturation of SPI by heat treatment could have resulted in a reduced interfacial tension between the oil and water phases, which could result in smaller droplets (D3,2) (Floury et al., 2000).

4.4.3.2 Effect of ultra high-pressure homogenization on oil droplets size of SPI emulsions

Chemical, pharmaceutical, specialty foods and biotechnology facilities all use the high-pressure homogenizer to emulsify, disperse, mix and process their products. Homogenization pressure is responsible for disruption of fat droplets and the creation of new interfaces (Boutin *et al*., 2007). The effect of high-pressure homogenization at 200-600 bars on SPI emulsions produced from heated and non-heated SPI dispersions was studied (Figure 4.4). High-pressure homogenization reduced droplet size of emulsions of both non-heated and heated SPI. The combination of heat treatment and high-pressure homogenization showed a larger reduction in oil droplet size of emulsions when compared

with the sample that was homogenized only (Fig. 4.4). High-pressure homogenization seems to break the non-covalent forces within insoluble protein aggregates, and make it easier for protein fractions to dissociate and escape from the insoluble protein aggregates to form soluble protein aggregates (Zheng *et al.*, 2008). High-pressure homogenization decreases oil droplets size because the oil and water mixture is subjected to intense turbulent and shear flow fields. Turbulence leads to the break up of the dispersed phase in to small droplets (Floury *et a*l., 2000), and a partial unfolding of protein occurs during homogenization. This involves breaking of physical interactions (loss of secondary structure) so that hydrophobic parts of the molecule are exposed and they facilitate protein anchoring at the interface, enhancing its emulsifying capacity (Kiokias *et al*., 2007). Over all this study shows that homogenization improved the stability of emulsions.

4.4.3.3 Effect of heat treatment of SPI in presence of polysaccharides on oil droplets size of SPI emulsions

Under all experimental conditions, heat treatment of SPI caused decrease in mean oil droplet size of emulsions (Figures 4.5 and 4.6). Heat treatment of SPI in the presence of glucose and or polysaccharides caused increase in oil droplet size. Results that homogenisation caused a significant decrease in droplets size (Figure 4.6). The presence of sugars (glucose/polysaccharides) in the aqueous phase of food systems containing SPI can alter the conformation and interaction of protein by binding to protein surface groups, or they may indirectly influence these characteristics by altering the physicochemical properties of water (Gu *et al.,* 2008). This could be because addition of polysaccharide enhanced the rate of emulsion droplet aggregation during heating, due to depletion flocculation, where emulsion droplets become aggregated by attachment of adsorbing macromolecules to more than one droplet at a time (Dickinson, 1992; Ye and Singh, 2006). The droplet size of emulsions made with SPI heated in the presence of both glucose and polysaccharides were larger than in presence of glucose or polysaccharides alone (Figures 4.5 and 4.6). This could be due to an increased sugar concentration, resulting in increased glycation. Gu *et al*., 2008 showed an increase in degree of glycation of SPI with sugars almost linear relative to sugar concentration, and reaching a plateau at concentration higher 20 %). Glycation of SPI was indeed demonstrated in Fig. 4.2.

The degree of glycation was not related to oil droplet size, since the highest glycation was obtained with pectin and lowest glycation was obtained with starch (section 4.2.2), while highest droplet size was obtained with locust bean gum.

4.4.4 Soy yogurts

In the acid gelation of soy protein, which is part of the manufacture of yogurt, the use of polysaccharides plays an important role in modifying the textural properties of the protein system (Abd karim *et al*., 1999). In this study texture profile analysis were used to assess the influence of glucose and/or polysaccharides on final texture parameters (hardness, cohesion, and adhesiveness) of soy yogurt. The textural properties of various compositions of soy yogurt are shown in Tables 4.1- 4.4. The addition of glucose or/and polysaccharides increased the gel hardness which could be attributed to the ability of sugars to form bonds with soy protein (Sabadini *et al*., 2006, Baeza *et al.*, 2002). These bonds would be strongly related to Maillard cross-linking which might increase the molecular entanglement in the gel structure, preventing the rupture of weak non-covalent interaction, and thus stabilize gel net work (sun *et al*., 2006). This result agrees with Peng *et al.*, (2000), and Campbell *et al.,* (2008) who found that Maillard reaction resulted in increased hardness of soy protein isolate-glucono-δ-lactone at pH 4.5 gels. In the present study the highest hardness was obtained by yogurts produced from SPI and glucose with pectin or amylopectin. Pectin and amylopectin showed the highest glycation with SPI (section 4.2.2). The lowest emulsion hardness with polysaccharides was obtained with starch because it has no reducing groups (Oste *et al.*, 1990). After heat treatment of yogurt samples followed by cooling down to room temperature, the hardness increased compared to before heat treatment. Mcklem, 2002; Campbell *et al.*, (2008) found an increase in intermolecular disulfide bonds in the gel matrix of that of GDL induced SPI gels. We show increased disulfide bonds in heated SPI (section 4.3.1), but we do not see this in yoghurts made with heated SPI, or in heated yoghurts (Tables 4.6 and 4.7). The present results show the primary bonds maintaining the gel network yogurt are hydrophobic bonds, while electrostatic and disulfide bonds were low in maintaining the yogurt structure (Table 4.7). The reason could be that at pH4.5 and in the presence of polysaccharides, sulfhydryl oxidation is rather limited, which is possibly the result of protein-polysaccharides formation, which reduces protein segment mobility and flexibility (Diftis and Kiosseoglou, 2006a). Dickinson and Merino-Matia (2002) reported that the direct contact between

protein and water is considered thermodynamically unfavourable in the presence of sugars, and this can be correlated directly with an enhancement of hydrophobic interaction through the modification of water structure surrounding the protein. Homogenization also increased hardness of yogurts (Table 4.2). Smaller oil droplets were produced by homogenization, which are immediately stabilized by a protein layer. It can be presumed that the smaller particles provided a better base for firmer protein network/aggregates to be formed (Atapattu, 1997).

The yogurt with the highest WHC was obtained from homogenized and heated soy protein isolate combined with glucose and pectin (Table 4.5), which was creamy without water separation. Pectin and glucose resulted in the highest water holding capacity; pectin being one of the most commonly used polysaccharides in acidic environments (Roudsari *et al*., 2006). This could be because pectin is an acidic polysaccharide and has a high retention of water at pH4.5 (Roudsari *et al.,* 2006). Carrageenan resulted in the lowest water holding capacity, probably due to enhanced gelation caused by electrolytic interaction with sulphate groups, (Molina Ortiz et al., 2004). Water holding capacity (WHC) is a quantitative indication of amount of water retained within a protein matrix under defined conditions (Huang and Kinsella, 1986). Since, the difference in glycation caused by carrageenan and pectin is relatively small 1.68 versus 1.62 µg/µl (Figure, 4.2), and the former resulted in poor WHC properties, whereas the latter results in excellent WHC. It can be concluded that the improvement in functionality if SPI-pectin and SPI-amylopectin complexes was not only due to the Maillard reaction, but also due to positive complex coacervation; and that phase separation caused by SPI-carrageenan was due to thermodynamic incompatibility as discussed in Chapter 1, section 1.10.4.3.

The present results show that glycated SPI gels have higher WHC than non-glycated gels, which could be due to a higher concentration of high molecular weight polymers taking part in the gel net work and resulting in better entrapment of water in the glycated SPI gels (Gu *et al.*, 2008). Addition of polysaccharides to soy yogurt could also increase WHC through gelation by increasing denser network, with smaller pores and greater capillary forces (Hua *et al.*, 2003; Maltais *et al*., 2005; Yamamoto and Cunha, 2007) and also by their ability to bind water with their hydroxyl groups (Uresti *et al.*, 2003). Heat treatment of yoghurts increased WHC, due to increased dissociation of oligmers and increased unfolding of protein, which lead to exposure of additional binding sites to water (Suliman *et al*.,

2006). These results agree with Gu *et al* (2008) who found glycated SPI has higher WHC than non-glycated SPI.

The second parameter measured with respect to textural analysis of soy yogurt was cohesion strength. Cohesion determines how much of a gel structure is destroyed after compression as related to force (Handa *et al.,* 1998). The results show for all yogurts tested, texture analysis indicated very low cohesion strength (Table 4.3). This could be because at low pH there is a reduction in stretch and cohesion strength, due to the more compact protein conformation (Lucey *et al.*, 2003). Adhesiveness, which was the third parameter measured, is defined as the work required to overcome the attractive forces between the food surface and surface of other materials which come in contact with food such as the plate, tongue, and teeth. It was expressed as a function of withdrawal force and time between two bits (Ju and Kilara, 1998). For all yogurts tested, texture analysis indicated very low adhesiveness (Table 4.4). This could be because adhesiveness depends on the balance of electrostatic and Van der Waal interaction and electrostatic interaction may play major role in adhesiveness (Boonaert and Rouxhet, 2000). At pH 4.5, which is the iso electric pH of SPI, electrostatic repulsive forces between protein molecules are decreased. As in this study in yogurt bonds (section 4.7) I have found electrostatic is low in maintaining the yogurt structure.

These results demonstrate that glycation of SPI with pectin or amylopectin, resulting in significanlt improved functionality, can be achieved in solution and not only by glycation in the dry state, as previous literature shows. We also showed increased disulfide bonds in heated SPI (Fig. 4.1) indicating that glycation occurred with denatured SPI. Very little has been reported in scientific literature of the effect of simultaneous denaturation and glycation of soy protein on its functional properties. Here we report for the first time, significant enhancement of functional properties of SPI, denatured and glycated with pectin or amylopectin.

4.5 Conclusions

To produce a soy yogurt with high quality (smooth, homogenous texture and high WHC), pre-treatment (heat treatment, addition of sugars and homogenization) is essential. The best yogurt obtained was with SPI heat-treated in the presence of glucose and pectin, which was characterized by highest WHC. The flow diagram below depicts the proposed process for manufacture of a high quality yoghurt (Figure 4.8):

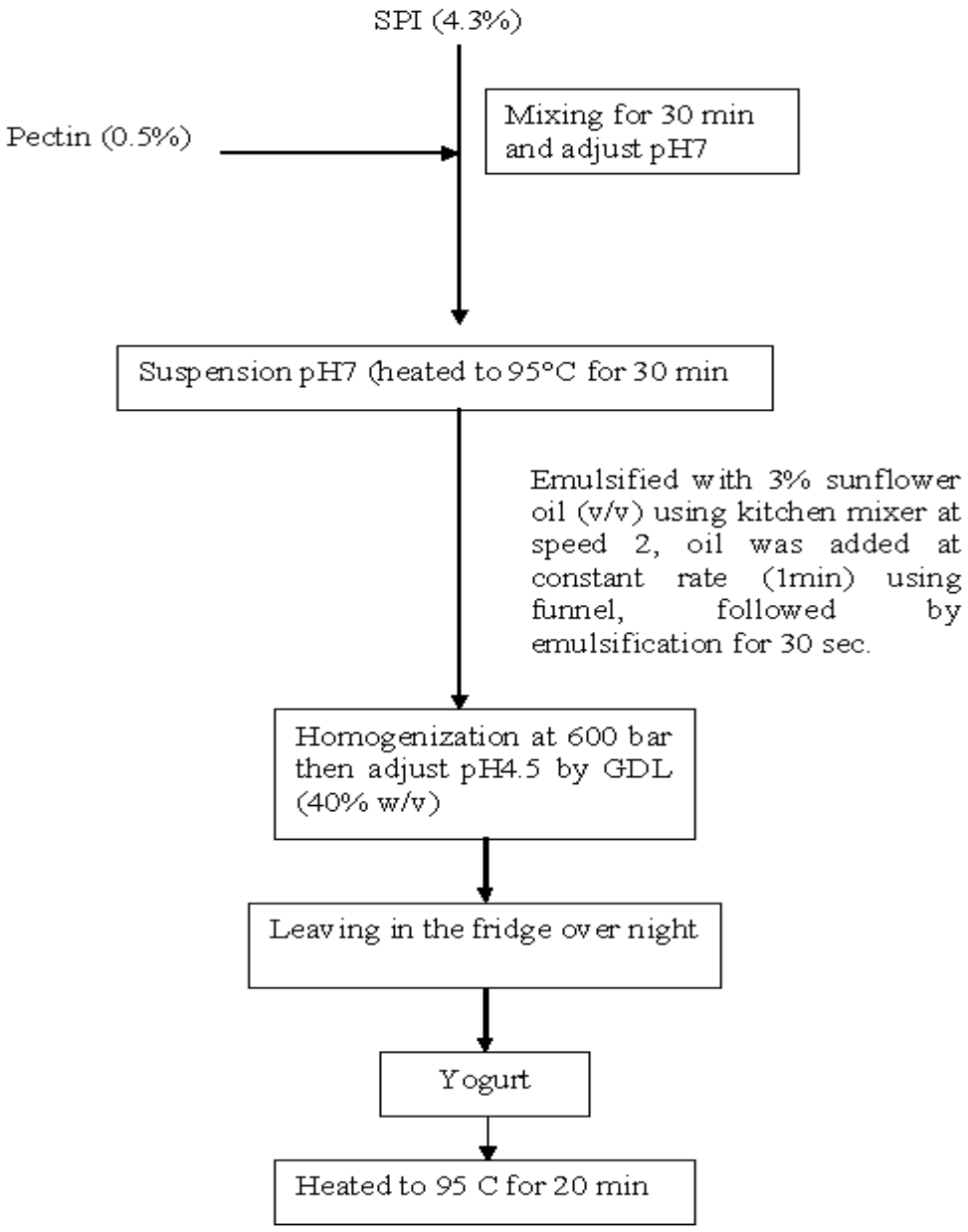

Figure 4.8 Flow diagrams proposed process for manufacture of a high quality yoghurt

Chapter five

Destabilization of SPI emulsions

5.1 Introduction

Many food products appear in the form of oil-in-water (o/w) emulsions, where the oil droplets are usually stabilised by proteins, acting as emulsifiers, along with other surfactants. Parameters of homogenisation, as well as the composition of the mixture, determine the physico-chemical properties of the emulsions (Sünder *et al*., 2001). To increase the shelf life of foods, products can be preserved with heat treatments to inactivate the spoilage microorganisms present. These pasteurisation or sterilisation treatments extend the shelf life but also affect product quality. This also accounts for food products that are based on emulsions, like infant formula, dressings and spreads. Heat treatment may result in flocculation of the oil droplets in the emulsions, resulting in phase separation. The effect of heat treatment on emulsion stability depends on the product composition, including factors like pH, ionic composition and the intensity of the heat treatment (Van de ven *et al*., 2007). Globular proteins (whey and soy proteins) are widely used as emulsifying agents in emulsion-based products because of their ability to facilitate emulsion formation and to improve emulsion stability. Globular proteins facilitate droplet disruption by reducing the amount of energy required to generate small droplets during homogenization. On the other hand, they improve the long-term stability of emulsions by generating repulsive colloidal interactions between droplets and by forming an interfacial film around the droplets that is resistant to rupture (Chanasattru *et al*., 2008). Emulsions are thermodynamically unstable because of the energy required to increase the surface area between oil and phase, and so they tend to separate into their component phases with time (Julian McClements and Dungan, 1995). Under some circumstances proteins are required components in fluid emulsions that also need to undergo extensive heat-treatment. An example is in the formulation of paediatric formulae for feeding to newborn/premature infants. This can lead to instability of the emulsion (Euston *et al.*, 2001). In this case understanding how the ingredients in the product (such as sugars) affect heat stability become very important. Information of this nature could allow for the optimisation of the heat stability of protein-stabilized emulsion through careful selection ingredients.

Soy proteins material is usually combined with polysaccharides, which increase the emulsion continuous phase, viscosity and improve the product rheological properties and texture characteristic. When however protein-stabilized emulsions are subjected to heat treatment, for pasteurization and sterilization purpose, they tend to aggregate. Change in heat stability that occur in the presence of different polysaccharides, most likely arise from

adsorbed layer composition and structure (Euston *et al*., 2001). The aim of this work is to a study of heat stability of emulsion system, containing SPI, whey, SPI plus polysaccharides (pectin, carrageenan and xanthan), SPI plus whey protein at different concentrations and temperature with different heating time, this allow manfactures to chose suitable ingredient to optimise emulsion heat stability. The objectives of this study have been achieved by completing a number of tasks.

Task 1 (SPI emulsions)

In this task, I have determined the rate of aggregation of SPI stabilized emulsions at different SPI concentrations and temperatures at pH4.5. The information gained will help us to understand better the mechanism of instability and aggregate formation in heated SPI emulsions. In addition, I have study rate of aggregation of whey stabilized emulsions at different concentrations and temperatures at pH4.5. The objective of this study was was to determine if interactions occurred between whey protein and SPI in emulsion system to increase its stability at different concentrations, temperature and different heating time at pH 4.5.

Task 2 (SPI- polysaccharide emulsions)

In the task I have investigated the effect different polysaccharides (pectin, carrageenan and xanthan) on heat stability of emulsion made with SPI, I have aimed to understanding and identifying particular ingredients and ingredient combination that may be used to optimise the heat stability of SPI stabilized emulsions at pH4.5.

5.2 Materials and Methods

Mechanisms of destabilization of SPI emulsions and whey emulsions, prepared as described in Chapter 2 section (2.2.3.3 and 2.2.3.4), using the kinetics of aggregation to describe the destability of emulsions by the equation of relative change in the number of emulsion droplets (N_t/No) as described in Chapter 2 section (2.2.8.2.1)

5.3 Results

5.3.1 Destabilization of SPI and WPC emulsions

5.3.1.1 Kinetic analysis of SPI emulsion heat destabilization

Heating a soy-protein stabilised emulsion will, depending on the pH and temperature, lead to aggregation of the emulsion droplets. This can be followed using a kinetic plot of assuming kinetics of order 1.5, as illustrated in Figure 5.1

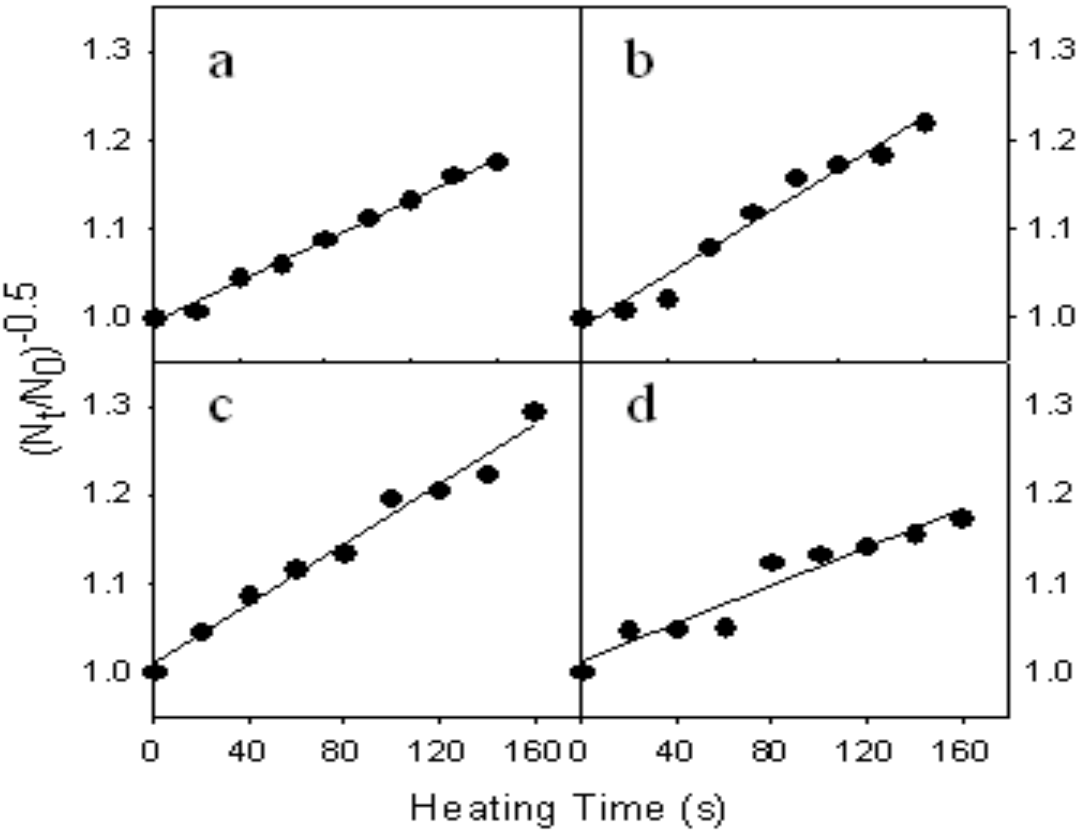

Figure 5.1 – Kinetic plots for soy-protein-stabilised emulsion made at pH 4.5 with varied concentrations of protein and heated at 100 ^{0}C for different time. (a) 0.75 (w/v)% soy protein; (b) 1.5(w/v)% soy protein; (c) 2.25(w/v)% soy protein; (d) 3(w/v)% soy protein.

The kinetic plots have been analysed by calculating the reaction rate constant, k, from the slope of kinetic plots like Figure5.1 using equation 2.2 (chapter 2; section 2.2.8.2.1). The rate constant was then corrected for differences in the initial droplet number.

5.3.1.2 Effect of pH on SPI emulsion heat stability

In Figure 5.2, I have plotted the value of k for emulsions at different pH and protein content and heated at different temperatures to 100 ^{o}C for 3min. It is immediately obvious that the heat stability behaviour of the emulsions was strongly dependent on the pH at which they are made.

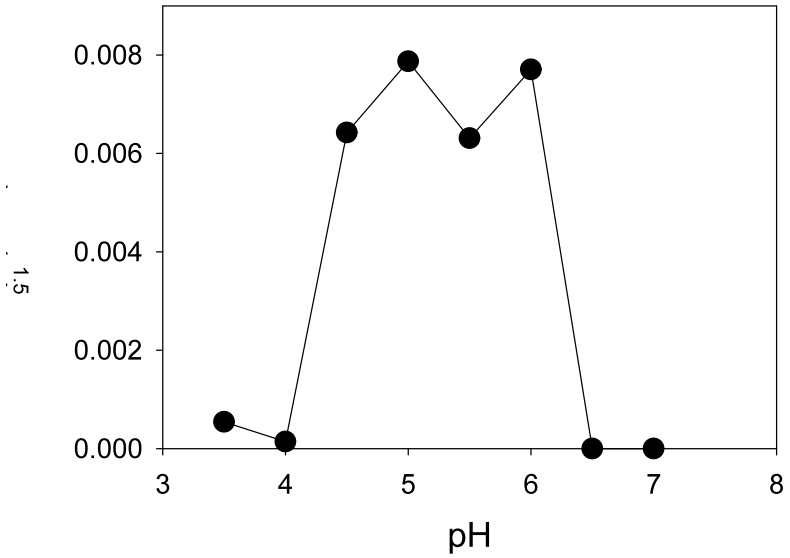

Figure 5.2 - Plot of rate constant for the heat-induced changes in soy protein stabilised emulsions heated at 100 ^{o}C for 3min as a function of pH. Protein concentration = 3 (w/v) %.

Figure 5.2 shows that there is a region between pH 4.5 and pH 6 where the emulsions are highly unstable. Above or below these pH limits the emulsions are highly stable to heating. The explanation for this behaviour lies in the solubility behaviour of soy protein as a function of pH.

5.3.1.3 Comparison of the concentration dependence of the heat stability of SPI and WPC emulsions

In addition to the pH dependence of SPI emulsion heat stability, I have also investigated the protein concentration dependence and the temperature dependence. For these systems, as emulsions are unstable upon heat tratment at a pH close to their pI, a pH

of 4.5 was chosen for these system. Figure 5.3 is a plot of the apparent rate constant for SPI emulsions heated at various temperatures at pH 4.5

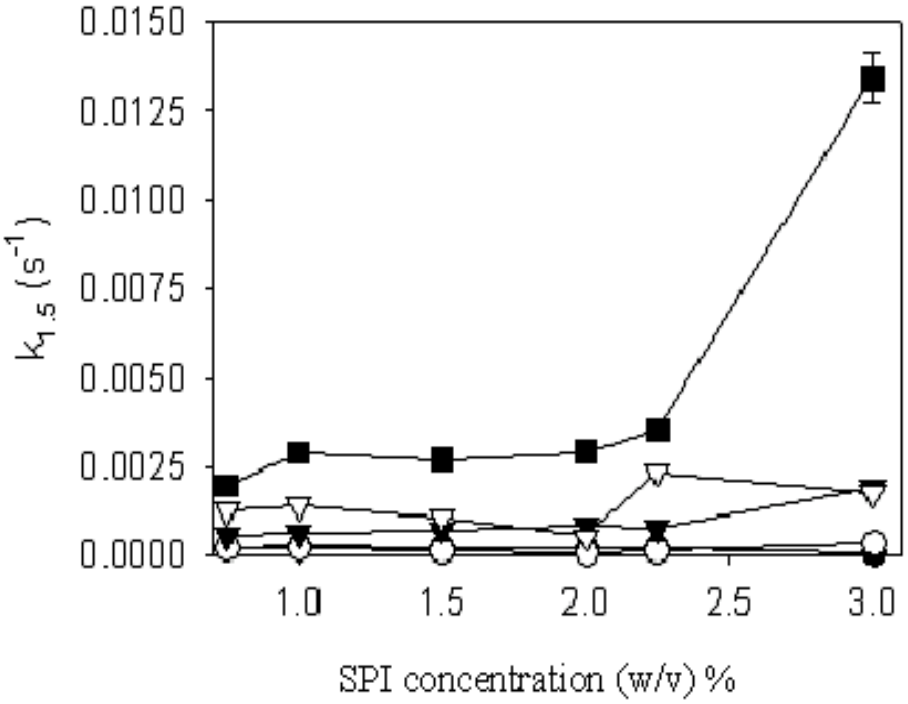

Figure 5.3 – Plot of apparent reaction rate constant for heat-induced destabilization of SPI emulsions (pH4.5, 20 (w/v)% sunflower oil) as a function of SPI concentration. The emulsions were destabilized by heating at various temperatures.

● 60 °C; ○ 70 °C; ▼80 °C; ∇ 90 °C; ■ 100 °C.

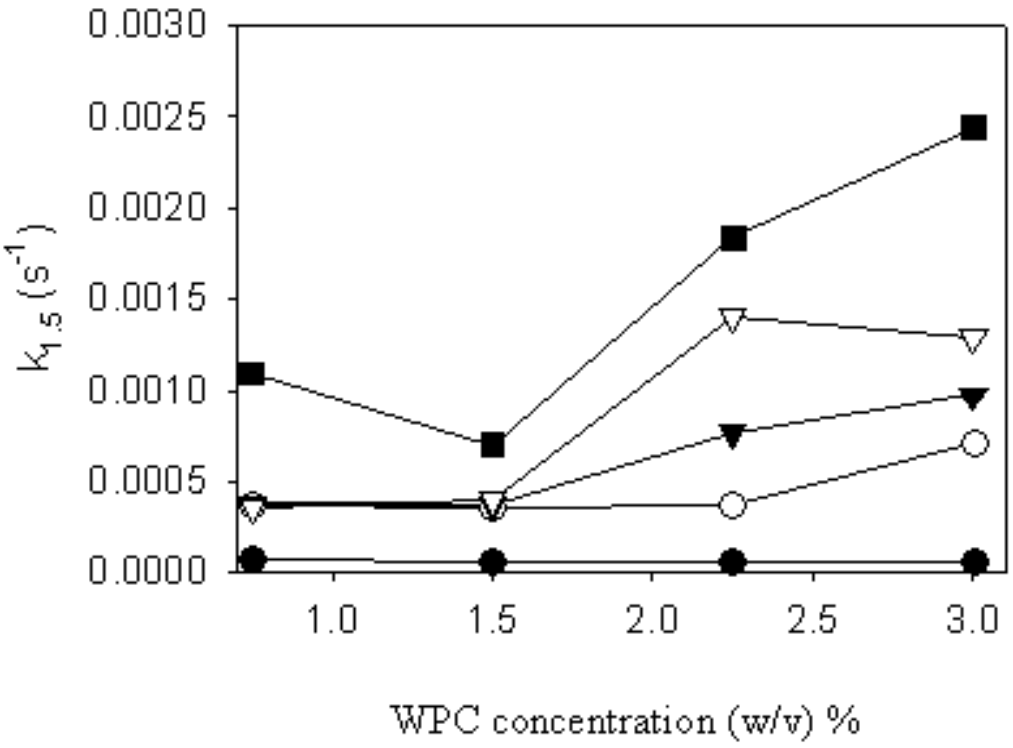

Figure 5.4 – Plot of apparent reaction rate constant for heat-induced destabilization of WPC emulsions (pH4.5, 20 (w/v) % sunflower oil) as a function of WPC concentration. The emulsions were destabilized by heating at various temperatures.

● 60 °C; ○ 70 °C; ▼80 °C; ∇ 90 °C; ■ 100 °C.

At temperatures below 100 °C the destabilization of SPI emulsions was only weakly dependented on protein concentration. Even at 100 °C saw a large increase in k can be observed when the protein content reached 3%. The behaviour of SPI emulsions to that of WPC emulsions heated under the same conditions is compaerd in Figure 5.4. Whey protein makes emulsions that are more stable to heating than SPI under the same conditions (i.e. k is lower for WPC emulsions at the same temperature/concentration combination). In addition, WPC emulsions show stronger concentration dependence.

5.3.1.4 Comparison of the temperature dependence of the heat stability of SPI and WPC emulsions

The temperature dependence of k for both SPI and WPC emulsions is illustrated in the Arrhenius plots of Figures 5.5 and 5.6. As expected, k shows a strong dependence on

temperature for both SPI and WPC emulsions at all protein concentrations. The Arrhenius plot shows an interesting difference between SPI and WPC stabilized emulsions.

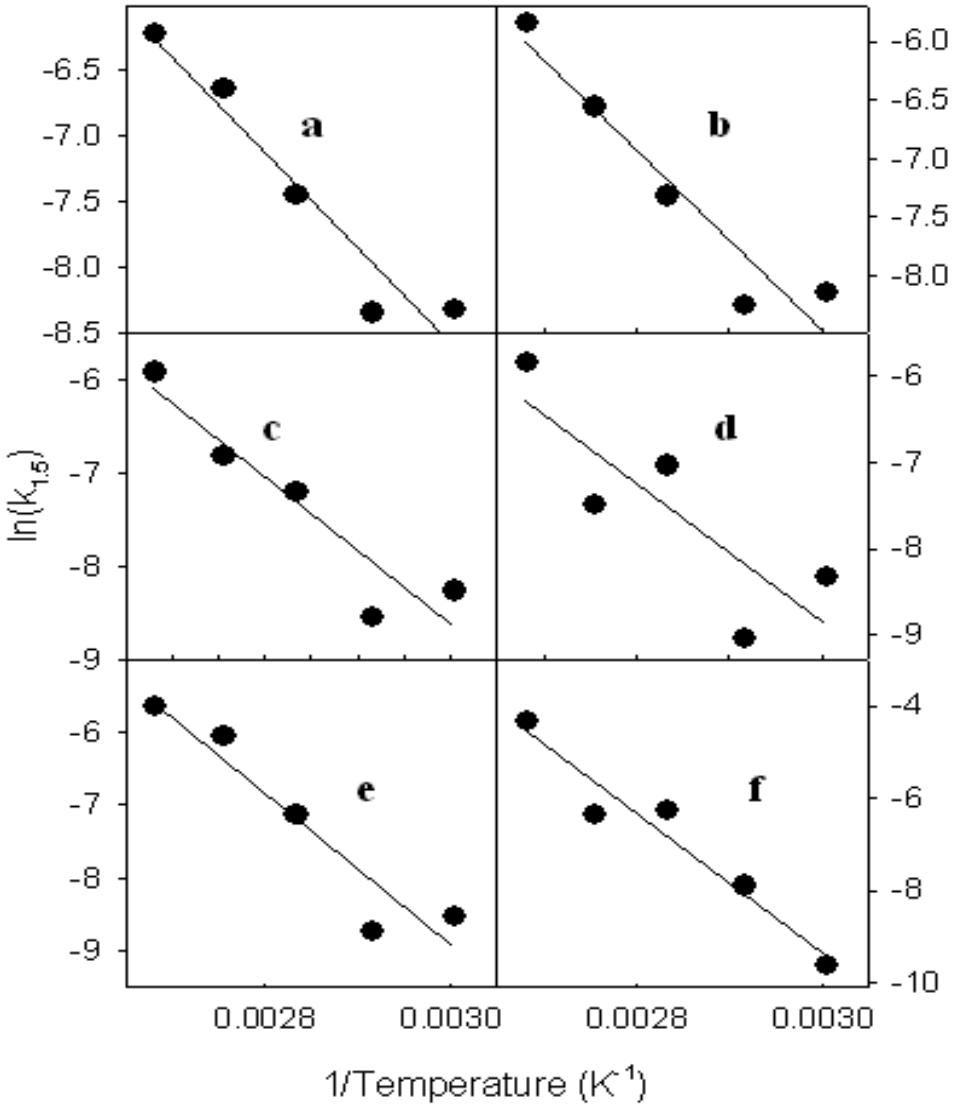

Figure 5.5– Arrhenius plots for SPI emulsions (pH 4.5, 20wt% sunflower oil). (a) 0.75 (w/v)% SPI; (b). 1.0 (w/v)% SPI; (c). 1.5 (w/v)% SPI; (d). 2.0 (w/v)% SPI; (e). 2.25 (w/v)% SPI; (f). 3.0 (w/v)% SPI.

The Arrhenius plots for SPI emulsions are linear (Figure 5.5), whilst those for WPC show the bilinear (5.6) behaviour typical of that observed for heat denaturation of WPC or β-lactoglobulin. The activation energy E_a can be calculated from the slope of the data in Figures 5.5 and 5.6, and compare to typical values calculated for denaturation of whey

proteins and soy proteins in solution. Similarly, the free energy, enthalpy and entropy of activation can also be calculated using equations (2.6-2.8; chapter 2; section 2.2.8.2.1)). These are shown in Table 5.1 for both SPI and WPC emulsions.

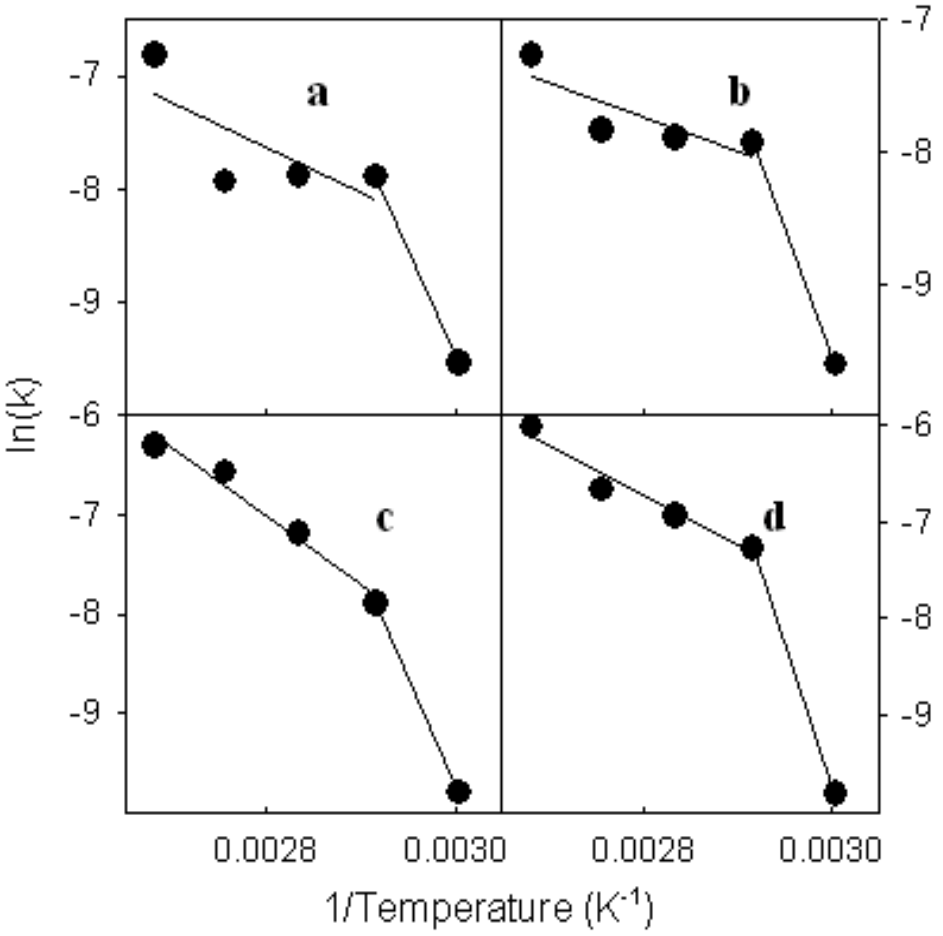

Figure 5.6 Arrhenius plots for WPC emulsions (pH 4.5, 20 (w/v)% sunflower oil).

(a) 0.75 (w/v)% WPC; (b). 1.5 (w/v)% WPC; (c). 2.25 (w/v)% WPC; (d). 3.0(w/v)% WPC.

5.3.1.5 Mixed SPI and WPC emulsions

The dependence of $k_{1.5}$ on the ratio of SPI:WPC in the emulsion was shown in Figure 5.7 for different heating temperatures. At low temperatures $k_{1.5}$ showed no significant dependence on SPI:WPC ratio. At higher temperatures, however $k_{1.5}$ increased as the proportion of SPI increased from 0% SPI (3% WPC) to 3% SPI (0% WPC). If one were to drawn a straight line between $k_{1.5}$ for 0% SPI and 3% SPI, the $k_{1.5}$ for intermediate mixed SPI/WPC emulsions, falls below this line. This suggested that the WPC component of the mixed emulsions is determined the heat stability behaviour of the emulsions. The temperature dependence of the emulsions with varied SPI:WPC compositions were shown in the form of Arrhenius plots in Figure 5.8. Somewhat surprisingly, the mixed emulsions showed a linear Arrhenius plot similar to SPI rather than the bilinear plots of WPC emulsions.

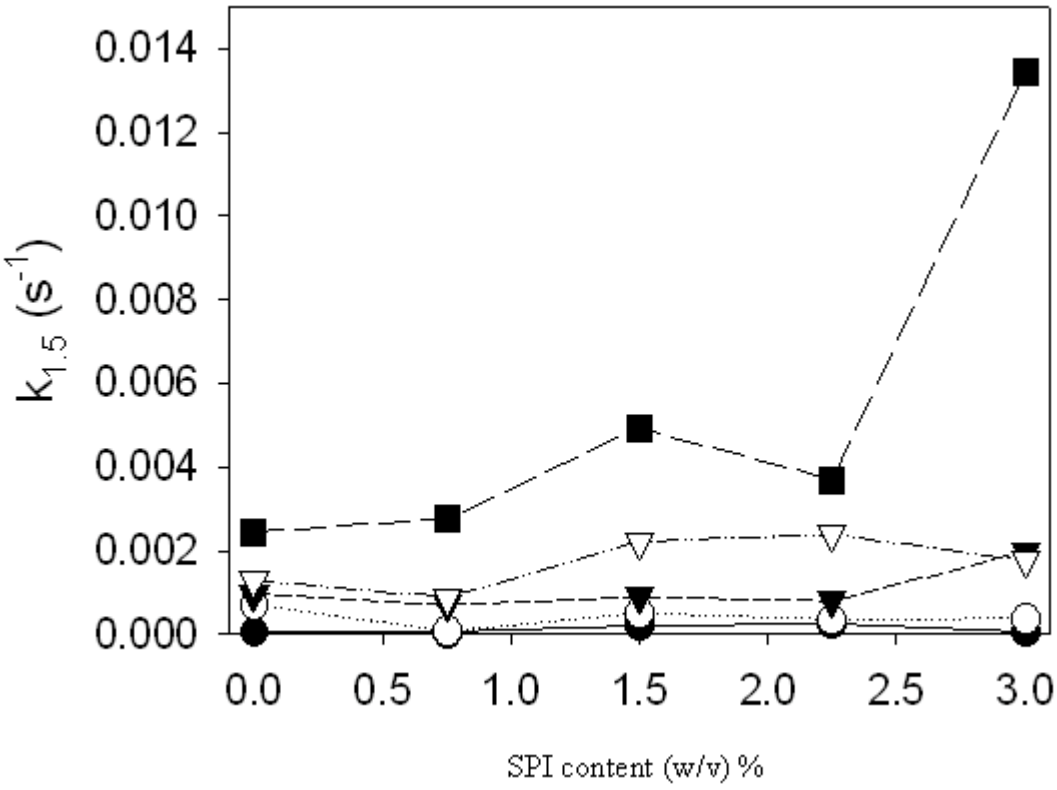

Figure 5.7 - Plot of $k_{1.5}$ for heat-induced destabilization of mixed SPI:WPC emulsions (pH4.5, 20(w/v)% sunflower oil, total protein content 3 (w/v)% as a function of SPI: WPC ratio. The emulsions were destabilized by heating at various temperatures.

● 60 °C; ○ 70 °C; ▼80 °C; ∇ 90 °C; ■ 100 °C.

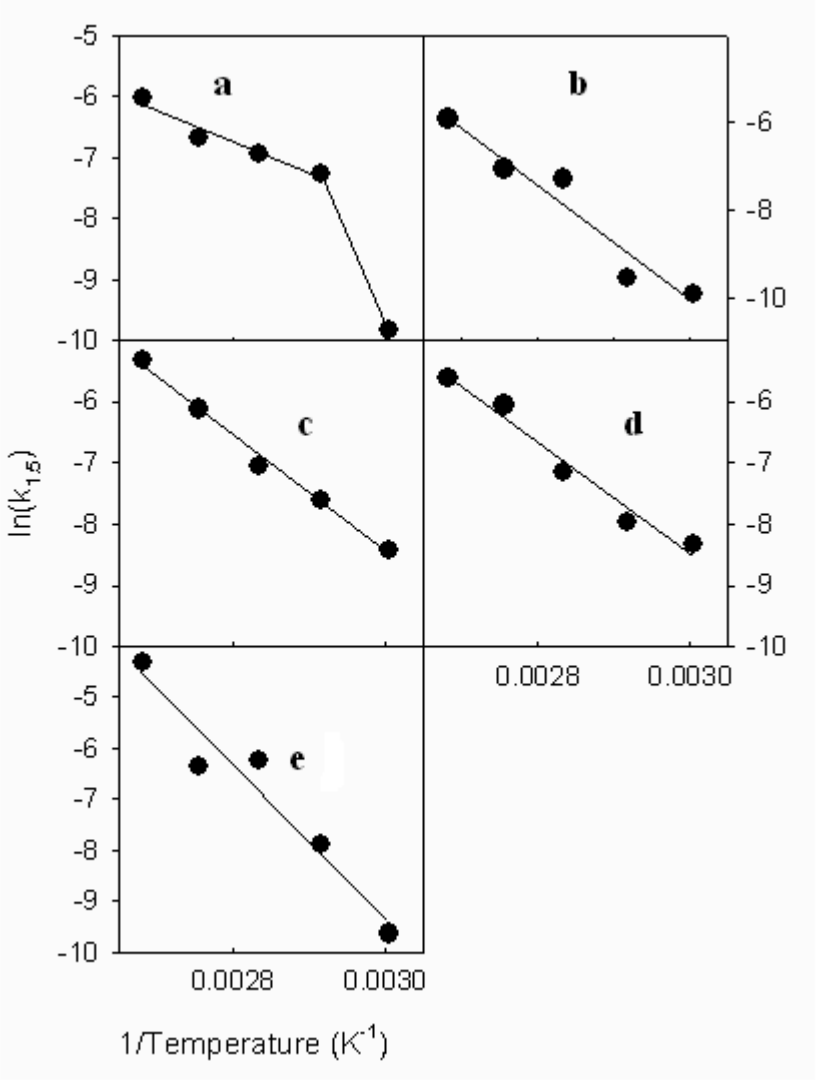

Figure 5.8 – Arrhenius plot for mixed SPI:WPC emulsions.

(a). 0% SPI, 3(w/v)% WPC; (b). 0.75(w/v)% SPI, 2.255 (w/v)%WPC; (c). 1.5(w/v)% SPI, 1.5(w/v)% WPC; (d). 2.25(w/v)% SPI, 0.75(w/v)% WPC; (e). 3(w/v)% SPI, 0% WPC.

5.3.2 The effect of polysaccharides on SPI emulsion heat stability

Addition of polysaccharides to SPI stabilized emulsions only appeared to have a large effect on the heat stability for the emulsions at higher temperatures (Figure 5.9). For all polysaccharide types, the most pronounced effect was a large decrease in the rate of destabilization when 0.01wt% of the polysaccharide was added to the emulsion that was heated at 100 ^{o}C.

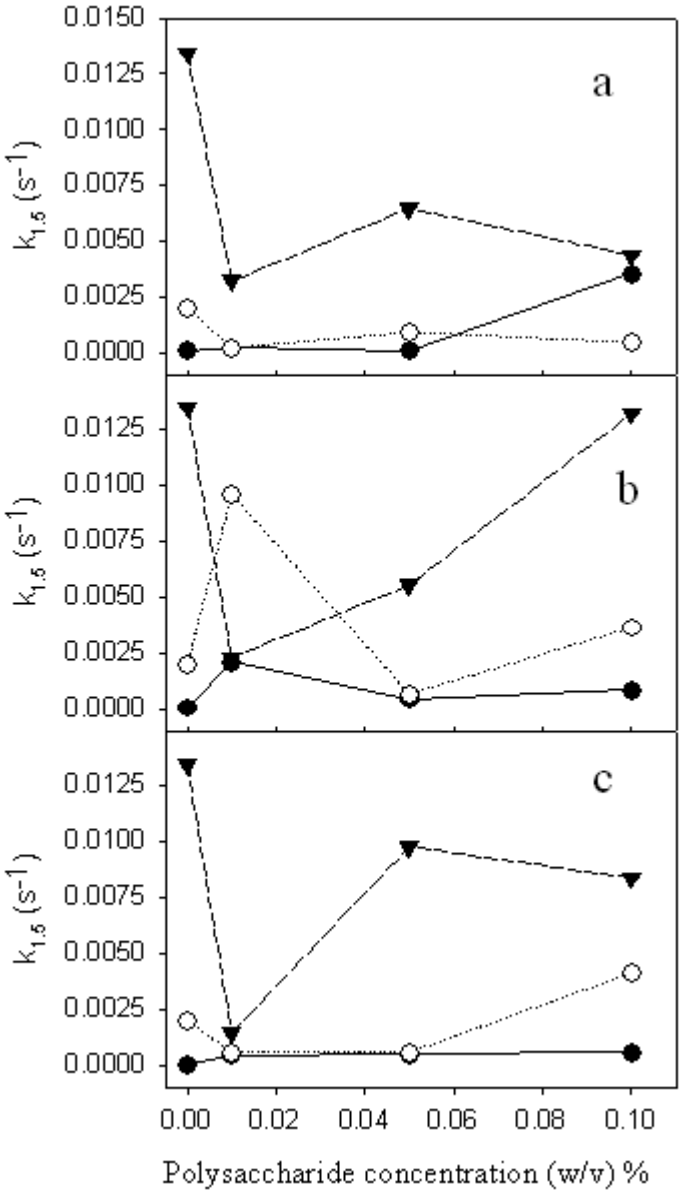

Figure 5.9 – Effect of polysaccharides on the heat stability of SPC emulsions. (a) pectin; (b). xanthan; (c) carrageenan.

● 60 °C; ○ 80 °C; ▼100 °C.

Table 5.1 Activation energy (E_a), free energy of activation (ΔG), enthalpy (ΔH) and entropy (ΔS) of SPI and WPC at different concentration and temperatures.

Protein Concentration	E_a (kJ mol^{-1})	T (^{o}C)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (kJ mol^{-1} K^{-1})
SPI 0.75%	61	60	105	58	-0.14
		70	108	58	-0.15
		80	109	58	-0.14
		90	110	58	-0.14
		100	111	58	-0.14
SPI 1.0%	64	60	104	62	-0.13
		70	108	61	-0.13
		80	108	61	-0.13
		90	109	61	-0.06
		100	110	61	-0.13
SPI 1.5%	66	60	105	63	-0.13
		70	109	63	-0.13
		80	108	63	-0.13
		90	110	63	-0.13
		100	110	63	-0.13
SPI 2.0%	67	60	105	64	-0.12
		70	110	64	-0.13
		80	108	64	-0.12
		90	112	64	-0.13
		100	110	64	-0.12
SPI 2.25%	87	60	105	84	-0.06
		70	109	84	-0.07
		80	108	84	-0.07
		90	108	84	-0.07
		100	110	84	-0.07
SPI 3%	126	60	108	123	0.04
		70	107	123	0.05
		80	105	123	0.05
		90	109	123	0.04
		100	105	123	0.05
WPC 0.75%	155	60	108	153	0.13
	33	70	107	30	-0.22
		80	110	30	-0.23
		90	113	30	-0.23
		100	113	30	-0.22
WPC 1.5%	159	60	108	156	0.14
	21	70	107	18	-0.26
		80	110	18	-0.26
		90	113	18	-0.26
		100	115	18	-0.26
WPC 2.25%	181	60	109	178	0.20
	57	70	107	55	-0.15
		80	108	55	-0.15
		90	109	54	-0.15
		100	112	54	-0.15

Continuation of Table 5.1

Protein Concentration	E_a (kJ mol^{-1})	T (^{o}C)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	*ΔS* *(kJ mol^{-1} K^{-1})*

WPC 3.0%	241	60	109	239	0.39
	42	70	105	40	-0.19
		80	107	39	-0.19
		90	110	39	-0.19
		100	111	39	-0.19
SPI 0%	241	60	109	239	0.39
WPC 3%					-0.19
	42	70	105	40	-0.19
		80	107	39	-0.19
		90	110	39	-0.19
		100	111	39	
SPI 0.75%	109	60	109	106	-0.01
WPC 2.25%		70	112	106	-0.02
		80	108	106	-0.01
		90	111	106	-0.01
		100	110	106	-0.01
SPI 1.5%	79	60	105	76	-0.09
WPC 1.5%		70	106	76	-0.09
		80	108	76	-0.09
		90	108	76	-0.09
		100	109	76	-0.09
SPI 2.25%	126	60	105	123	0.05
WPC 0.75%		70	107	123	0.05
		80	108	123	0.04
		90	108	123	0.04
		100	109	123	0.04
SPI 3%	126	60	108	123	0.04
WPC 0%		70	107	123	0.05
		80	105	123	0.05
		90	109	123	0.04
		100	105	123	0.05

5.4 Discussion

5.4.1 Destabilization of SPI and WPC emulsions

Studies (Lakemond et al., 2000b; Chove et al., 2007; Pizones Ru´ız-Henestrosa *et al,* 2007) have demonstrated that the solubility of soy protein isolate and individual 7S and 11S globulins show a rapid decrease in solubility around the iso-electric point of pH 4.5. The solubility curves for soy protein reported by these authors mirrored very closely the behaviour I have observed for emulsion heat stability in that high solubility corresponded to high heat stability and *vice versa*. The high heat stability at neutral pH for soy proteins as compared to other globular proteins was attributed to their ability to denature and form soluble complexes (German *et al*., 1982; Sorgentini *et al.*, 1995). Renkema *et al*., (2000), showed that both *β*-conglycinin and glycinin were denatured on heating SPI to 95 °C at neutral pH and that disulphide interchange reactions occurred between acidic and basic polypeptides of glycinin. This led to the formation of soluble complexes between basic polypeptides of glycinin and *β*-subunits of *β*-conglycinin. In contrast, when SPI was heated at acidic pH (3.8–4.5) most of the protein became insoluble, with no evidence of formation of soluble protein complexes (Renkema *et al.*, 2000; Renkema *et al.*, 2002). Obviously, when emulsions pH were adjusted after homogenization, those that were adjusted to a pH close to the pI would tend to aggregate. In their aggregated form, these emulsions were more susceptible to heat-induced destabilization. The results for the rate constant $k_{1.5}$ with those of Anema & McKenna (1996) can be compared. Who have studied whey protein denaturation in reconstituted whole milk. A direct comparison of rate constants was not appropriate, as this study has used used relative initial droplet number to calculate $k_{1.5}$ in equation (2.3) whilst Anema & McKenna, (1996) used the relative protein concentration, but a comparison of trends in the results is valid. Anema & McKenna (1996) found that the rate denaturation of β-lactoglobulin, the major protein component of whey, was relatively unaffected by protein concentration. The same was true for both SPI and WPC emulsions in this study over much of the concentration range studied (Figures 5.3 and 5.4). In Figure 5.4, WPC emulsion stability did appear to be dependent on concentration, but the actual changes in $k_{1.5}$ were relatively small and only appeared large due to the scale used.

Of course, the presence of oil droplets in this study will complicate the mechanism of heat-induced destabilization. The net effect of these is to act as filler particles that occupy a volume of the system that the protein will be excluded from. The protein covered oil droplets act as if they were large protein particles, and increase the effective concentration of the proteins. A comparison this results for WPC stabilized emulsions with those of Anema and McKenna (1996) is therefore complicated by the apparent differences in the

whey protein concentrations, and also the differences in the aqueous phase used (imidazole buffer, pH 4.5 in case of this study and milk serum at its natural pH of close to 7 for Anema & McKenna, (1996). The protein content of milk varies between 2.2%-4.4% (Euston, 2008) of which about 20% is whey protein (Smithers, 2008). Therefore, one might expect the milks made by Anema & McKenna (1996) to have whey protein content somewhere in the range 0.45-0.88%, i.e. lower than the range of protein contents used in this study. One must absorved that much of the protein in emulsions used in this study were adsorbed to the oil droplet surface, and this increased the apparent protein concentration, possibly to as high as 20%. One might therefore expect different concentration dependence for results of this study compared to those of Anema & McKenna (1996).

A comparison of SPI emulsion heat stability with the denaturation of soy proteins in solution is hindered by their being a lack of studies comparable in thoroughness to those of Anema & McKenna (1996). For whey proteins, Ahmed *et al*. (2006) reported a significant change in the rate of gelation of SPI when the concentration was raised from 10% to 15%. Again, direct comparison with results of this study is difficult due to the complicating effect of the oil droplets and differences in the methodology used to determine k.

The Arrhenius plots for SPI and WPC emulsions revealed some interesting differences. The most noticeable difference was that $k_{1.5}$ for SPI emulsions falls close to a straight line over the full temperature range from 60 – 100 ^{o}C, whilst the results for WPC emulsions were better fitted to a bilinear plot with $k_{1.5}$ for 60 ^{o}C falling considerably below the extrapolation of the straight line fit for the other temperatures. To explain these differences between SPI and WPC we should consider the composition of the two protein powders. WPC is composed predominantly of β-lactoglobulin (β-lac) (50%), α-lactoalbumin (α-lac) (20%) with the remainder made up of immunoglobulins, lactoferrin, lactoperoxidase, serum albumin lysozyme, and other minor peptides (Smithers, 2008). In WPC made from cheese only, 15% of the total whey is made up of glycomacropeptide (Smithers, 2008), the peptide derived from κ-casein which is non-globular and therefore heat stable. When heated both β-lac and α-lac denature, but unlike β-lac, α-lac does not rapidly aggregate due to a lack of free thiol groups (Dalgleish et al., 1997). Thus, one might expect the heat stability of emulsions in this study to be dominated by the β-lac component of the WPC. This is consistent with the bilinear Arrhenius plots in Figure 5.7. The change in slope of the Arrhenius plots for pure whey proteins occurs at the denaturation temperature of the

protein, which for β-lac occurs between 80-85 ^{o}C (Anema & McKenna, 1996). Interestingly, for emulsions in this study this break occured at a lower temperature around 70 ^{o}C. One should remember that in an emulsion much of the protein would be adsorbed to the oil-water interface and will therefore be in a partially surface denatured state before heating. The results suggested that surface denaturation made the protein more susceptible to heat-induced instability, and lowered the temperature required to initiate significant heat-induced breakdown to 70 ^{o}C rather than the 80-85 ^{o}C required for pure β-lac solutions.

Soy protein isolate is more heterogeneous in its composition than is WPC. SPI is comprised of two major fractions, β-conglycinin or 7S globulin (30%) and glycinin or 11S globulin (35%). Both of these of these denatures, albeit at a different temperature, and contribute to the heat-induced aggregation of SPI. The denaturation temperature for 11S globulin is above 90 ^{o}C at pH 4.5, whilst that of 7S globulin is around 72 ^{o}C. Therefore, when SPI is heated the denaturation and aggregation behaviour will be a composite of the behaviour of the major protein components. For SPI emulsions in this study, this led to a linear Arrhenius plot since the denaturation temperatures for the different soy protein components were sufficiently far apart that they contributed to heat destabilization in different regions of the temperature range studied. When WPC and SPI mixed in emulsions, the Arrhenius plots also showed linear rather than bilinear behaviour. This is because addition protein added to the mixture, making it more heterogeneous. β-lactogobulin has a denaturation temperature of 85 ^{o}C, mid way between that of 7S and 11S globulins.

One can compare the activation energies calculated from the Arrhenius plots of Figure 5.6 for WPC emulsions with the results of Anema & McKenna (1996) for β-lac. The values of E_a fell in the range 155-241 kJ mol^{-1} for the low temperature range, and 21-57 kJ mol^{-1} for the high temperature range. Anema & McKenna, (1996) found an E_a of between 263-296 kJ mol^{-1} and 33-51 kJ mol^{-1} for the low and high temperature ranges respectively depending on the genetic variant of β-lactogobulin (A or B variant). Their values were comparable to those found by Dannenberg & Kessler (1988a and 1988b) reported in an earlier study. Values of this study were broadly comparable to those of Anema & McKenna, (1996) suggesting that the reactions leading to heat-induced aggregation of the emulsion droplets were the same. However, the fact those activation energies were slightly lower than those of Anema & McKenna (1996) may be a consequence of the partial denaturation of the adsorbed protein.

There are few results for the E_a of soy protein solutions with which to compare results in this study. However, those those are available revealed large differences between the E_a for SPI emulsions and solutions. Añón and coworkers (Petruccelli & Añón, 1996; Scilingo & Añón, 1996) reported E_a values of around 160-200 kJ mol^{-1} for 7S globulin and between 270-440 kJ mol^{-1} for 11S globulin, with these values being strongly pH dependent for 11S globulin. These values were considerably higher than the values found for emulsions prepared in this study, which were in the range 61-126 kJ mol^{-1}. This values were much closer to those determined by Ahmed *et al.,* (2006) where E_a was found to vary between 129.8 and 35.5 kJ mol^{-1} for a 10% and 15% (w/w) solution of SPI respectively, and the results of Yoshi et al., (1990) where E_a was found to be in the approximate range 40-105 kJ mol^{-1} for a range of powders with moisture contents between 0% and 30%. The different values of E_a obtained in these studies could be partly due to the different methodologies used to determine them. Alternatively, it may point to the soy proteins in emulsions prepared in this study have already significantly been denatured when they were adsorbed at the oil-droplet surface. In addition, the E_a obtained for soy protein denaturation appeared to be highly dependent on both pH and protein concentration which complicated comparison of results in this study with other solution studies which tend to be carried out at or close to neutral pH and at high protein concentrations (typically 10 wt% and above).

For the other parameters determined using equations (2.6)-(2.8) a similar comparison can be made with results in the literature. The ΔG values for samples in this study were all in the range 104-115 kJ mol^{-1} for both WPC and SPI emulsions. This was consistent with Anema & McKenna's (1996) results for whey protein. In fact, it is believed that the value of ΔG may be relatively constant at around 100 kJ mol^{-1} based on the study by Labuza (1980) who reported this value for 18 different proteins.

The enthalpy of activation (ΔH) is a measure of the energy enthalpy barrier for the reaction. It was similar to the activation energy (Table 5.1); I can see that the values of E_a and ΔH are almost the same. For WPC emulsions prepared in this study, ΔH (153-239 kJ mol) was slightly lower than that found by Anema & McKenna (1996) (260-290 kJ mol^{-1}) for temperatures below the change in slope of the Arrhenius plot, and comparable for temperatures above the change in slope (18-55 kJ mol^{-1} for emulsions and 30-55 for β-lac in solution). No data for SPI solutions could be found for comparison. It seems from these results that when the whey proteins were adsorbed to the oil droplets surface the enthalpy

of activation for heat-induced aggregation was reduced, probably because the proteins were already partially denatured before heating commenced.

Inspection of the relative values for E_a, ΔH and ΔS can tell one something about the mechanism of the heat destabilization reaction for protein stabilized emulsions. The mechanisms of protein denaturation and aggregation is believed to occur in two stages where in the first stage the protein initially denatures, and then in the second stage the proteins aggregate to form a precipitate or a gel if the concentration is high enough. A similar mechanism is likely to underpin the heat instability in globular protein stabilized emulsions. One or other of these reactions will determine the overall rate of the reaction. If the denaturation step is rate limiting, it is expected that E_a and ΔH will be high and ΔS positive. The high values of E_a and ΔH are a consequence of the energy that must be put into the system to overcome the interaction forces holding the protein tertiary structure together. A positive ΔS indicates that on denaturation the structure of the protein becomes more disordered. The opposite is true if the aggregation step in the reaction is rate limiting, i.e. E_a and ΔH will be lower since aggregation occurs, due to the formation of bonds between the proteins, and the conformational restriction on the protein that occurs when it is aggregated means it occupies a smaller number of conformations, is more ordered and gives a negative ΔS. One can see that for WPC emulsions E_a and ΔH were high and ΔS wass positive at 60 ^{o}C (Table 5.1), indicating that denaturation was slow and limits the heat-induced destabilization of the emulsions. At 70 ^{o}C and above, E_a and ΔH were low and ΔS was negative, which suggested that the denaturation of the protein was fast and the aggregation step was rate limiting. These results are consistent with those of Anema & McKenna (1996) who also found that for whey protein heat instability, the denaturation step is limited at temperatures below the denaturation temperatures of the proteins, and the aggregation step limiting at temperatures above this.

This result of SPI emulsions showed a behaviour that was characteristic of an aggregation limited reaction under most of the conditions studied, i.e. a low E_a and ΔH and negative ΔS. It was only when the SPI concentration reached 3% that one could see an increase in E_a, ΔH and positive ΔS, suggesting a denaturation limited reaction. These results were unusual, and suggested that the heterogeneity of protein composition of SPI led to a very complex thermal behaviour.

The mixed SPI/PC emulsions showed an even more complex dependence of thermodynamic parameters on emulsion composition. As a forementional, 3% WPC emulsion showed a change denaturation to aggregation when the temperature increased to 70 ^{o}C. Low additions of SPI to the emulsions led to a combination of thermodynamic parameters characteristic of an aggregation limited reaction at all temperatures. However, as the proportion of SPI:WPC was raised to 3:1 and pure 3% SPI the heat-induced destabilization became denaturation limited at all temperatures.

5.4.2 The effect of polysaccharides on SPI emulsion heat stability

The effect of polysaccharide on the stability of protein stabilised emulsions is complex. Many food products contain both polysaccharides and proteins. In particular, mixtures of proteins and polysaccharides can be found among the ingredients of a wide range of colloidal food systems such yogurts, mayonnaise and ice cream (Neirynck et al., 2007b). The effect of polysaccharides on emulsion stability is complex (Dickinson & Pawlowsky, 1997). Under some circumstances, polysaccharides will increase the stability of emulsions. This can occur through a simple increase in viscosity, or through specific interactions between adsorbed proteins and polysaccharide molecules. Under other conditions, polysaccharides destabilize emulsions by inducing flocculation. This is called depletion flocculation and is caused by localized imbalances in osmotic pressure in mixed emulsions/polysaccharides system. As a result, there is an osmotic flow of water from the region between two droplets out into the bulk aqueous phase. The net result is a shrinking of aqueous phase volume between the droplets and a net force pulling the droplets together. If the droplets are closer together at the time of heating, this in itself should be enough to increase the likelihood of aggregation (Euston *et al.*, 2000). According to Reiffers-Magnani et al., (2000); Euston *et al*., (2002) the proteins that were adsorbed at surface of fat globules were “colloidal proteins” and could be thermodynamically incompatible with polysaccharides as it was for free proteins.

The results of this part of the study showed that polysaccharides increased stability of SPI emulsions under most conditions of temperature and protein concentration (Figure 5.9). This is an unexpected result. Destabilization of SPI emulsions appears to occur mainly via a coalescence mechanism and addition of polysaccharides to emulsions enhanced the coalescence of the emulsion droplets, probably by causing depletion flocculation (Dickinson, 1992; Ye and Singh, 2006). Creaming of SPI emulsions in the presence of

polysaccharide, which suggested they were flocculated. For some reason, however, this did not lead to an increase in the heat instability of the SPI emulsions, as one would expect. Emulsion in this study, pectin appeared to have a protective effect (Figure 5.9a). Bonnet *et al*., (2005), have observed that pectin was able to stabilise casein emulsions against acid gelation in the presence of glucono-δ-lactone. It is likely that a similar effect occurred in SPI emulsions in this study and this led to the increase in heat stability. For xanthan gum (Fig. 5.9b), an initial decrease in $k_{1.5}$ at low xanthan concentration and then an increase in $k_{1.5}$ back up to the value when no xanthan was present (Figure 5.9b), was seen as polysaccharides concentration was increased to 0.1%. This behaviour was different to what has been observed in study of Euston et al., (2002), with WPC emulsions, where the apparent rate constant increased as the concentration of xanthan is increased. This was attributed to depletion flocculation, a similar behaviour with carrageenan was absorved (Fig. 9c). This too differed from previous results with WPC stabilised emulsions and carrageenan where depletion flocculation led to an increasing k as the polysaccharide concentration was increased (Euston et al., 2002). Carp *et al*., (1999), have found that xanthan was capable of altering the composition of the soy protein adsorbed layer in foams containing SPI and xanthan. More specifically, xanthan appeared to promote the aggregation of soy protein at the interface, and increased the surface viscosity. It is possible that a similar effect occurred in SPI emulsions in this study, and it is likely that any alteration of the composition and stability of the adsorbed protein layer will lead to changes in the susceptibility to heat instability. In this case, if the adsorbed layer contained more protein in an aggregated state, which gave a higher surface viscosity this led to increased resistance to heat induced denaturation and aggregation (Figure 5.9b).

With SPI emulsions it has been found by Molina Ortiza et al., (2004) that in the protein-polysaccharide complexes were formed in the presence of carrageenan. This had the effect of causing the proteins to denature at higher temperatures, as evidenced by DSC thermograms. This might be sufficient to make SPI emulsion more stable to heating when carrageenan is present.

It appeared that soy proteins behave differently in the presence of polysaccharides than do whey proteins. These differences in the interactions between polysaccharides and the two types of proteins led to different susceptibility to heat-induced destabilization.

5.5 Conclusions

The taskes of this study study showed in

Task 1 (SPI and WPC emulsions)

(I) study showed that emulsion heat instability was weakly dependent on protein concentration at temperatures below 100 ^{o}C.

(II) Instability increased with increasing temperature.

(III) The kinetics of the destabilization of SPI emulsions is complex, and shows a linear Arrhenius plot, unlike the bilinear plots of WPC.

(IV) Addition WPC to SPI emulsions increases their heat stability.

(V) The mixed WPC and SPI emulsions again show a complex behaviour, with linear Arrhenius behaviour similar to that of SPI emulsions

Task 2 (SPI- polysaccharide emulsions)

The results of this part of the study show that polysaccharides increased stability of SPI emulsions at low concentrations, with higher additions having less of an effect on heat stability, but still not decreasing the heat stability. This in contrast to the findings in the literature for WPC emulsions where polysaccharides decreased heat stability at all levels of addition. This is explained in terms of the known interactions of various polysaccharides with soy proteins, and the effect this has on emulsion stability or soy protein denaturation.

Chapter six

General Conclusion

6.1 Summary of tasks

`The fundamental aim of this study was to evaluate the effect of heat treatment combined with glycation on functional properties of soy protein.

The underlying approach for improvement of protein functional properties was by heat-induced, non-enzymatic interaction with various sugars and polysaccharides. To support this, the effects of parameters such as heating temperature, protein concentration and various sugars and polysaccharides were investigated. I have tried to improve the functional properties of two commercial types of soy proteins (1) Soycomil K obtained from ADM, which is an insoluble kind of soy protein concentrate sold as animal feed, and (2) soy protein isolate obtained from ADM. I have prepared native SPC and SPI on laboratory scale to compare the structural modifications of commercial soy proteins. The main functional properties of proteins tested were solubility, emulsifying ability (oil droplet size), water holding ability, gelling ability in yoghurt system and heat stability in an acidic emulsion. In addition, the effect of SPI on the heat stability of whey protein in acidic emulsions was investigated, the reason being that whey protein is a common ingredient of acidified dairy emulsions such as yoghurts, where problems due to instability during pasteurisation are often encountered. This study was conducted by completing three tasks:

6.2 Summary of results

6.2.1 Functional properties of SoyComil K

Physicochemical studies of SoyComil K showed that it had a denaturation degree of 28% and that hydrophobic and disulfide bonds were the main bonds maintaining its structure. Its low solubility compared to other commercial soy protein concentrate (Arcon ® SJ, manufactured in the USA), and soy isolates appeared to be due to significantly higher disulfide bond content as shown by SDS-PAGE. It is assumed that this was due to the use of alcohol (ethanol) in the extraction process during manufacturing (chapter 1 section 1.5.2). Many treatments were applied to SoyComil K to increase its solubility (heat treatment, change in pH and protein concentration, treatment with enzymes, and addition of sugars). The study shows:

6.2.1.1 Heat treatment in the absence of sugars

The highest increase in solubility was obtained by heat treatment of 6% SoyComil K, pH9 at 100 °C for 10 min, the solubility increased from 8.42% to 30%, while at 80°C for 10 min was obtained with 0.1M NaCl (6% SoyComil K) at pH 9, the solubility increased from 8.42% to 9.6%.

6.2.1.2 Heat treatment in the presence of sugars

The highest increase in solubility was obtained with heat treatment of 6% protein pH 6.5 with sucrose (1%), at 70°C for30 min. The solubility increased from 2.44% to 7%. The highest degree of glycation was obtained with glucose and lowest was with sucrose. Under these heating conditions (70°C for30min) glycation increased the solubility of Soycomil (chapter 3 sections 3.3.2.4 and 3.3.2.4.1).

At high heating temperature (100°C), glycation caused a decrease in solubility probably due to increased covalent protein cross-linking via Maillaird reaction.

6.2.1.3 Enzymes treatment

The highest increase in solubility (12.82%) was obtained with α-amylase after heat treatment (6% soyComil K pH 9) at 80°C for 10 min, this could have been due to removal of a protective layer of insoluble soy polysaccharides which co-purified with the protein during the manufacturing process (chapter 1 section 1.5.2) rendering it more susceptible to heat treatment. However mixing with α-amylase for 24 hours followed by heat treatment at 100°C decreased the solubility (from 30% to 24 % compared to control), due to increased hydrophobic interactions. Treatment by proteinase K increased solubility (20.8%) of SoyComil K (6% at pH9) at 80 °C for 1hour. This could be due to the enzymatic release of smaller polypeptide units. However, treatment by proteinase K followed by heat treatment at 100°C decreased the solubility (from 30% to 20.4% compared to control), due to increased hydrophobic interactions.

6.2.1.4 SoyComil K emulsions

SoyComil K treated with glucose resulted in emulsions with the smallest average droplet size (section 3.3.3), indicating improved emulsifying ability. Since Soycomil treated with glucose had the highest glycation degree, it can be concluded that

increased glycation with glucose correlated with increased emulsifying ability and water holding capacity.

6.2.1.5 Rheological measurements (emulsion viscosity)

Emulsions of heated SoyComil K solution (SoyComil K emulsions as prepared in section 2.2.3.1) had under all circumstances a higher viscosity than non-heated emulsions.

6.3 Soy protein isolate and polysaccharides in model yogurt

An optimal process was developed for the preparation of yoghurt prepared with SPI with smooth texture and no water separation, comparable to a commercially available yoghurt prepared with soy milk.

- Soy protein isolates (SPI) used in this study had a denaturation degree 25%.
- Intermolecular bonds of non-heated SPI molecules were mainly electrostatic, whilst in heat treated SPI, they were mainly hydrophobic bonds and disulfide bonds
- Pectin and amylopectin resulted in the highest degree of glycation of SPI (available amino group 1.62 μg/ μl and 1.63 μg/ μl respectively), whereas the lowest glycation degree was obtained with starch (available amino group 1.7 μg/ μl). Were the values of available amino group for pectin and amylopectin significantly less than starch at Confidence levels were set at 95% ($n=3$, $p<0.05$). No development of yellow or brown colour was visually noticeable in any of the samples, therefore it is concluded that these results reflect glycation in the early stage of Maillard reaction.
- The degree of glycation of SPI was not related to oil droplet size of the emulsions. The .The largest droplet size was obtained with SPI heated in the presence of locust bean gum, which had 1.65 μg/ μl available amino groups, while highest glycation was obtained with starch (available amino group 1.7 μg/ μl), but has droplets size smaller than emulsion from SPI and locustbean. Were the values of available amino group for locustbean significantly less than starch at Confidence levels were set at 95% ($n=3$, $p<0.05$).
- Heat treatment of SPI before emulsification, decreased the average droplet size of the emulsions.
- High-pressure homogenization reduced droplet size of all emulsions.

- The presence of glucose/polysaccharides and heat treatment increased droplets of all emulsion produced from homogenized or non-homogenized SPI dispersions.
- Yogurts produced from SPI and sugars (polysaccharides and glucose) showed that the microstructures (droplet size) are fairly homogeneous and more condensed compared to control (yogurt from SPI only).
- Hardness of yoghurts correlated with the glycation degree, where highest hardness was obtained with pectin.
- Homogenization increased hardness of yogurts.
- The yoghurt with the highest WHC was obtained from homogenized and heated soy protein isolate combined with glucose and pectin, which was creamy without water separation. Carrageenan resulted in the lowest water holding capacity, probably due to enhanced gelation caused by electrolytic interaction with sulphate groups. pectin significantly has water holding capacity more than carrageenan at Confidence levels were set at 95% ($n=3$, $p<0.05$)
- In general, glycated SPI gels resulted in higher WHC than non-glycated gels, which could be due to a higher concentration of high molecular weight polymers taking part in the gel net work and resulting in better entrapment of water in the glycated SPI gels.
- Because the difference in glycation degree between SPI/pectin and SPI/carrageenan was relatively small (but statistically significant), it is concluded that complex coaservation of pectin and SPI contributed to enhanced WHC, whereas thermodynamic incompatibility of carrageenan and SPI resulted in phase separation, hence reduced WHC.
- The present study show the primary bonds maintaining the gel network yogurt is hydrophobic bonds, while electrostatic and disulfide bonds were low in maintaining the yogurt structure. The reason could be that at pH4.5 and in the presence of polysaccharides, sulfhydryl oxidation is rather limited.

6.4 Destabilization of SPI and WPC emulsions

To increase the shelf life of foods, products can be preserved with heat treatments to inactivate the spoilage micro-organisms present. When however protein-stabilized emulsions are subjected to heat treatment, for pasteurization and sterilization purpose, the proteins tend to aggregate due to protein denaturation. It was shown in the previous section that the presence of glucose/polysaccharides of heat treated SPI also resulted in increased

droplet size of emulsions. In this section I have studied three cases with the aim to understand the mechanisms of destabilization of SPI emulsions.

6.4.1 Destabilization of SPI and WPC emulsions

The results show**:**

- Emulsion heat instability was weakly dependent on protein concentration at temperatures below 100 ^{o}C.
- Instability increased with increasing temperature.
- The kinetics of the destabilization of SPI emulsions is complex, and shows a linear Arrhenius plot, unlike the bilinear plots of WPC.
- Addition WPC to SPI emulsions increases their heat stability.
- The mixed WPC and SPI emulsions again show a complex behaviour, with linear Arrhenius behaviour similar to that of SPI emulsions

6.4.2 The effect of polysaccharides (pectin, carrageenan and xanthan) on SPI emulsion heat stability at different temperatures at pH4.5.

The results of this part of the study show that polysaccharides increased stability of SPI emulsions at low concentrations, with higher additions having less of an effect on heat stability, but still not decreasing the heat stability. This in contrast to the findings in the literature for WPC emulsions where polysaccharides decreased heat stability at all levels of addition. This is explained in terms of the known interactions of various polysaccharides with soy proteins, and the effect this has on emulsion stability or soy protein denaturation.

6.5 Novel findings

In the present study, the following novel findings were made, which have not been previously reported in the literature:

1) The insolubility of Soycomil was shown to be due to increased disulfide bond content

2) The solubility of Soycomil K was increased by heat treatment at certain protein concentration and pH and by amylase digestion

3) Emulsifying ability of SoyComil K was improved as shown by increased viscosity and water binding of emulsions

3) Soy protein, heat treated and glycated in solution with pectin or amylopectin, resulted in increased hardness and WHC in yoghurts. Most examples in scientific literature relate to increased functionality of food proteins glycated in the powdered state (at low water content), which indicates glycation of non-denatured proteins. The results in the presents study show improved functionality for soy protein, denatured and glycated simultaneously in solution.

4) A novel non-dairy yoghurt was developed containing SPI, glucose and pectin, matching the texture of commercially available yoghurt made with soy milk.

5) We have studied for the first time the thermodynamics of heated emulsions Arrhenius plots for WPC emulsion show atypical bilinear behaviour, whilst for SPI they are linear. We believe this is due to the cooperative effect of more than one denaturing protein in SPI emulsions, whilst for WPC emulsions denaturation and aggregation behaviour is dominated by β-Lactoglobulins. We have shown that the activation energy for heated WPC and SPI emulsions is lower than for WPC and SPI solutions. This suggests some pre-denaturation of the protein at the oil-water interface.

6.6 Future work

The following studies need to be carried out in the futher:

1. Modification of extraction process of SoyComil K production to give denatured soluble protein.

2. Investigate heat stability of more complex food emulsions containing more than two ingredients (protein, polysaccharide and low molecular emulsifier).

3. Use mixed polysaccharides to improve functionality of soy protein isolate in model yogurt.

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